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A Comparison of the Reduction of Alginic Acid by Different Methods

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A COMPARISON OF THE REDUCTION OF ALGINIC ACID BY DIFFERENT METHODS

A thesis submitted by

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SUMMARY

Several workers have reduced acidic polysaccharides for structural studies, for sorption studies, and for studies on chemical reactivity. All these investigators have used reduction procedures which have not been extensively studied and do not completely reduce the acidic groups. In addition, it is not known to what extent the other functional groups such as esters and hemiacetal are reduced. The goal of the present study is to obtain a further understanding of the reduction with both a Lewis acid, diborane, and a Lewis base, lithium borohydride, by comparison of the percent reduction of the functional groups on an acidic polysaccharide.

Alginic acid from the stipes of the brown algae Laminaria hyperborea was selected as the acidic polysaccharide for study. It is readily obtained in high yield as a linear 1-4, linked polyuronide of high molecular weight, composed of D-mannopyranouronic and L-gulopyranouronic acid units in the ratio of 1:3 (M/G). A polyuronide rich in L-guluronic acid was selected because upon reduction the polysaccharide contained gulose, the sugar most reactive to acid hydrolysis. Thus, any depolymerization occurring during reduction would be magnified over what would occur for a polymer composed of a less reactive sugar.

Lithium borohydride was used to reduce a soluble methyl di-O-propionyl alginate and an insoluble partially reduced algin polymer. Diborane, generated both in situ and externally, was reacted with the soluble di-O-propionyl alginic acid. These four algin polymers were analyzed both before and after reduction to obtain figures for the percent reduction of the uronic acid carboxyl to a primary alcohol, the percent reduction of n-propionyl ester with cleavage to two alcohols and without cleavage to n-propyl ether, and the percent reduction of the hemiacetal end group to form an alditol.

A comparison of the percent reduction of the functional groups on algin polymers, shows that there is a marked difference in reduction with a Lewis base, lithium borohydride, and a Lewis acid, diborane. Toward lithium borohydride the order of reactivity of the functional groups is the following: hemiacetal end group > reductive cleavage of n-propionyl ester > uronic acid carboxyl. In contrast, toward diborane the order is reversed: uronic acid carboxyl > reductive cleavage of n-propionyl esters > reduction of hemiacetal end group. In addition, only diborane reduces a portion of the n-propionyl ester to an n-propyl ether. These results are explained by the hypothesis that the reduction with the nucleophilic borohydride involves transfer of the hydride ion to the electron-deficient center of the functional group, whereas reduction with the electrophilic diborane involves attack at the centers of the highest electron densities.

A comparison of the reduction of soluble and insoluble algin polymers with lithium borohydride has shown that insolubility has lowered the percent reduction of all the functional groups, but to different extents. This result is believed due to insolubility increasing the activation energy for reduction of all the functional groups.

The number average degree of polymerization (DP) of the di-O-propionyl alginic acid before reduction was 158. After reduction with diborane generated externally, the DP dropped to 111. After reduction with lithium borohydride, the DP dropped from 158 to 103. The polymer reduced with diborane generated in situ had a DP of 66. When diborane is generated in situ, boron trifluoride is present in the polymer solution. The boron trifluoride, being a strong Lewis acid, probably hydrolyzes the glycosidic bonds.

In order to achieve complete reduction of an acidic polysaccharide without having ethers linked to the reduced polysaccharide, the lithium borohydride reduction procedure is recommended. This procedure does, however, also completely reduce the hemiacetal end groups to alditol end groups. When the reduction of the hemiacetal end group is not desired, reduction by the external generation of diborane would be advisable. This procedure has the disadvantages of forming ethers in the reduced product and causing incomplete reduction of the uronic acid carboxyl groups.

INTRODUCTION

Studies of the relative reactivity of a number of representative groups toward the reductant diborane indicates the following order of reactivity: carboxylic acids > aldehydes > esters (1). On the other hand, toward alkali metal borohydrides the order of reactivity is the following: aldehydes > esters > carboxylic acids (2). Analysis of the products from reductions in which two soluble organic components competed for a limited amount of reductant was used to obtain the relative reactivities. It is known, however, that the reactivity of any functional groups can be greatly modified by the organic structure to which it is attached (2).

Many investigators have reduced acidic polysaccharides by a number of procedures. The best results, in terms of total acids reduced, were obtained by the reduction with diborane generated either externally or *in situ* and with lithium borohydride reduction of esterified acids. However, the polysaccharide is more reactive for reduction with either diborane or lithium borohydride if the polysaccharide is in solution (3,4). Solubility in the ether-type solvents used in the reduction has been achieved by acetylation or propionation[1] of the polysaccharide hydroxyl groups. Hence, the polysaccharide has carboxylic acid, ester, and aldehyde (hemiacetal)[2] groups available to react, yet no one has studied the extent to which each of these functional groups is reduced. In fact, most investigators, interested in the reduction of the carboxylic acids, have simply ignored the reactivity of the other functional groups.

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[1] Propionation is the term used to indicate acylation with propionic anhydride forming a n-propionyl ester.

[2] Although the C-1 hydroxyl group originally is substituted with an n-propionyl group, reductive cleavage of the ester linkage during the course of the reaction results in a hemiacetal end group.

The importance of these other functional groups can best be illustrated by examples.

REDUCTION OF ACIDIC POLYSACCHARIDES

Two groups of workers have shown that some of the ester groups on a polysaccharide are reduced to ether groups when an excess of diborane is generated *in situ*. Hirst, *et al*. (5) reduced di-*O*-propionyl alginic acid with diborane generated *in situ* by the addition of boron trifluoride to a solution of polysaccharide and sodium borohydride in bis (2-methoxyethyl) ether. The polysaccharide from the reduction was de-esterified by adding alkali to pH 10 and heating at 60-70°C. for two hours. The polysaccharide from the reduction had 91% of its uronic acid carboxyls reduced and contained 5.2% *n*-propoxyl as determined by the specific method of infrared spectroscopy (7). This polysaccharide was subjected to a graded acid hydrolysis for a study of structure. The presence of ethers and acids decreased the yield of the desired hydrolysis products so only the most abundant structural units could be identified. In an analogous manner, Ross and Thompson (6) reduced a fully acetylated 4-*O*-methylglucuronoxylan with diborane generated *in situ*. The "apparent methoxyl" content of the reduced polymer had increased from 2.19 to 4.84%. By using the gas chromatographic technique of Miller, Samsel, and Cobler (8), they were able to show that the reduced polymer had both ethoxyl and methoxyl groups in the approximate ratio of 2:1 (E/M). The ethoxyl content is believed to arise from the reduction of acetyl groups.

Prior to this work, McKee and Dickey (9) had reduced a fully acetylated 4-*O*-methylglucuronoxylan with diborane generated *in situ*. The de-esterified polysaccharide from the reduction was not analyzed for ethers. He subjected the reduced polysaccharide to a graded acid hydrolysis. From the yield of the

different oligosaccharides from both the reduced and unreduced polysaccharides, he proposed a mechanism for acid hydrolysis. Even though the presence of ethers on McKee's reduced polysaccharide should not alter his thesis conclusions, it may have altered his yield of unetherified hydrolysis products.

In order to avoid the formation of ethers, Walker (10) added sodium borohydride to a solution of fully acetylated 4-O-methylglucuronoarabinoxylan in tetrahydrofuran and reacted the polymer by bubbling gaseous diborane into the solution. Sodium borohydride was added to the reaction flask not to enhance the reactivity of the polymer but rather to prevent high acidity and possibly hydrolysis of the polysaccharide. Under the conditions of the reaction, sodium borohydride probably does not reduce esters or carboxylic acids (2). Using this procedure, he achieved two of his objectives: (a) the ether content of the polysaccharide did not change upon reduction and alkaline de-esterification, and (b) the molecular weight of the reduced polymer was the same after reduction. He, however, was able to achieve only 60% reduction of the uronic acid carboxyls. This low percent reduction is probably due to the sodium borohydride forming a sodium carboxylate which is not reduced with diborane (2). The sorption of the unreduced and partially reduced polysaccharides was compared on cellulose fibers.

In order to achieve more complete reduction, Ross and Thompson (6) reduced 4-O-methylglucuronoxylan, acetylated fully, with diborane gas. After reduction, the deacetylated polymer had 90% of its carboxyls reduced and a 20% lower methoxyl content. The hot alkaline depolymerization of the reduced and unreduced polysaccharides were compared. They did not consider, however, the reduction of the hemiacetal end of the polysaccharide. If the end of the polysaccharide molecule is reduced to an alditol, it is greatly stabilized to the action of hot alkali (11). How much of the end group was reduced and what influence it has on the conclusions is not known.

As an alternative to diborane reduction, Rees and Samuel (4) have developed a procedure involving the reduction of methyl esterified uronic acid carboxyls on acetylated polyuronides with lithium borohydride in boiling tetrahydrofuran. They reported that the reduction yields a polysaccharide containing "only a trace of uronic acid carboxyl." Again, the reduced polysaccharide was analyzed only for carboxyl content.

Apparently, no one has conducted physicochemical studies to determine the effect of uronic acid carboxyl groups upon molecular configuration, rheological properties, or sorption of polysaccharides. Any subsequent reduction should not only be specific for carboxyl groups but it should not degrade the polysaccharide. Changes in molecular weight would adversely affect any conclusions that could be drawn from such a study.

SELECTION OF ALGINIC ACID POLYSACCHARIDE

For a study of the different reduction procedures, the unreduced acidic polysaccharide should meet the following criteria:

1. The polysaccharide should be readily obtainable in high yield in very pure form. The presence of protein, esters, ethers, or lignin on the unreduced polysaccharide would make interpretation of the analysis difficult.
2. The polysaccharide should be one whose structure has been extensively studied and shown to be linear β 1→4 linked. In a linear polysaccharide, all the linkages should be more nearly of equal reactivity.
3. The polysaccharide should have no low molecular weight polymers so nearly quantitative recovery may be obtained in any step of the procedure. In this way, the change in molecular weight would be

real and not due to preferential loss of low-molecular-weight material.

4. The polysaccharide should be reactive to acid and alkaline hydrolysis in order to magnify depolymerization in any step. In this way the small effect of the reductant may be noticed.
5. The polysaccharide should be very high in acid content so small differences in total reduction may be easily ascertained.

Alginic acid from the stipes of the brown algae Laminaria hyperborea meets all these requirements!

THE STRUCTURE AND USE OF ALGINIC ACID

Alginic acid constitutes the principal carbohydrate in brown algae. It is a polyuronide composed of D-mannopyranouronic acid (12) and L-gulopyranouronic acid (13,14) moieties, the relative ratio of which varies in different species. The species Laminaria hyperborea* was chosen for the present study because the stipes of this algae reportedly contain alginic acid with an L-guluronic acid content of 75% (16). Reduction of this alginic acid yields a polysaccharide rich in gulose, the sugar most reactive to acid hydrolysis (15). The number average degree of polymerization has been found to be from 200 to 860 by osmotic pressure measurements (17). Partial fractionation into gulurone- and mannurone-rich materials has been done (18), but fractionation into two homopolymers has not been achieved.

Alginic acid readily yields fibers, and this together with x-ray diffraction measurements on algin fibers (19-21) provides evidence that this polymer is linear.

*In certain references (14,31) this species is also called Laminaria cloustoni.

The results from methylation studies (14,22-23) and periodate oxidation studies (24,25) all suggest that the uronic acids are connected in large part by 1→4 linkages. No evidence has been presented to show that there are other linkages or branching on the alginate molecule.

Partial acid hydrolysis of alginic acid to molecules with a degree of polymerization of 15 and fractionation of these hydrolysis products has yielded two fractions, one containing only L-guluronic acid and the other containing only D-mannuronic acid (26). This suggests that the two uronic acids are not randomly linked but rather exist in groups of at least 15 anhydrouronic acids of either L-guluronic or D-mannuronic acid.

Evidence for the 1→4, linkages in alginic acid being β for D-mannuronic acid is based upon isolation of oligosaccharides from the partial acid hydrolysis of a reduced alginic acid. Hirst, et al. isolated a crystalline mannobiose (4-O-β-mannopyranosyl-D-mannopyranose) and a mannogulose (4-O-β-D-mannopyranosyl-L-gulopyranose) (5). The β-linkage in the mannogulose is inferred from its negative rotation. Still unknown is the type of anomeric linkage on the gulose moiety. The strong negative rotation of sodium alginates ($[\alpha]_D = -130°$) indicates that the L-guluronic acid units are α-linked. The suggested structure of alginic acid is seen in Fig. 1.

Alginic acid has some commercial importance to the paper industry. The United States paper industry uses 950 tons of alginic acid per year (27). Alginic acid is usually used as its water-soluble sodium salt as an adjuvant in adhesives for corrugating medium (28,29), in the surface sizing of paper (27-30), and in pigment coatings (28-30).

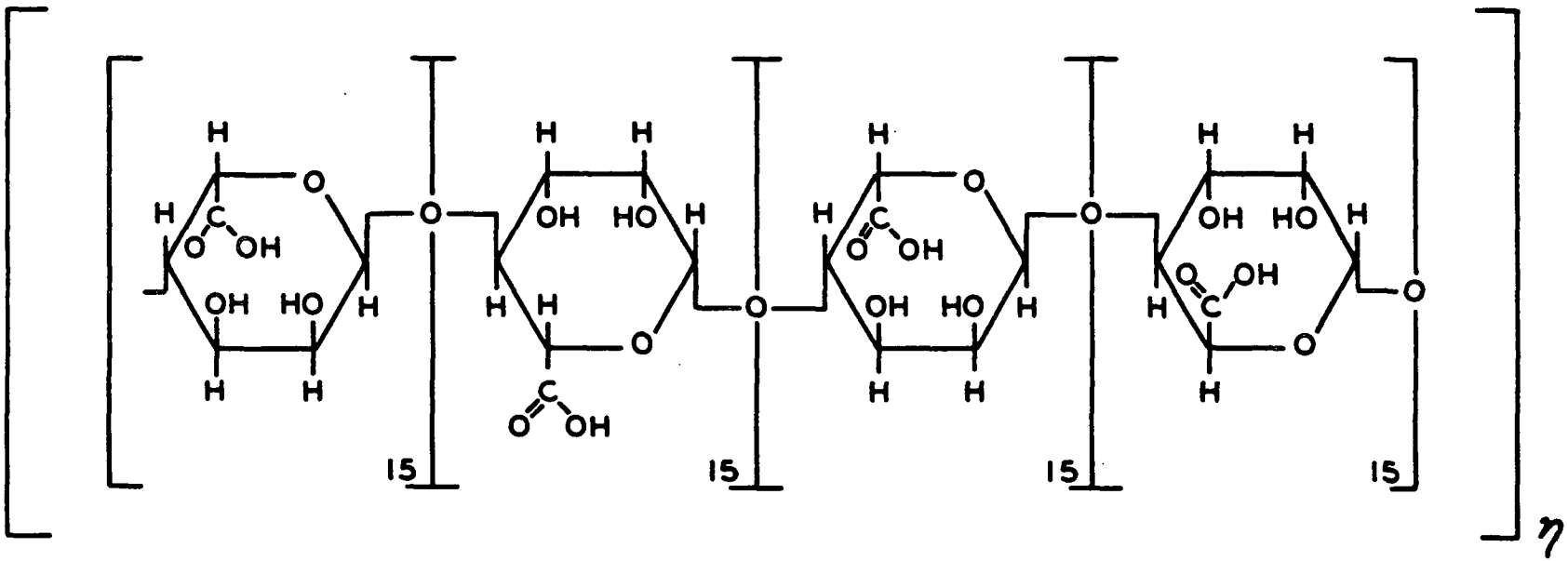


Figure 1. The Structure of Alginic Acid from the Stipes of Laminaria hyperborea

EXPERIMENTAL RESULTS AND DISCUSSION

PREPARATION OF ALGINIC ACID

Alginic acid was extracted from the stipes of the brown algae Laminaria hyperborea under the mildest possible conditions (31). Since the resulting product was gray and smelled of decaying meat, it was bleached by a very mild room-temperature acid chlorite treatment which is an adaptation of the procedure of Thompson and Kaustinen (32). The salts were removed from the bleached alginic acid by dialysis. The alginic acid was recovered as a white sponge by a new process called freeze-exchange (see page 54).

The composition of the hydrolyzed products from alginic acid was determined by the paper chromatographic procedure of Fisher and Dörfel (13). Their solvent systems are the only ones reported to separate guluronic acid from mannuronic acid or guluronic lactone from mannuronic lactone. From visual inspection of the intensity of the uronic acid spots and applying the correction of Haug and Larsen (33) for hydrolysis loss, the alginic acid was found to be composed of 75% guluronic acid and 25% mannuronic acid.

This analysis was complemented by using the phenol-sulfuric acid color reaction on sodium alginate from the L. hyperborea stipes. Haug and Larsen (33) have observed that "The colour development in the phenol-sulphuric acid colour reaction is different for the different uronic acids.....This reaction gives a colour corresponding to approximately 100% of the theoretical value when carried out on alginic acid not subjected to hydrolysis.....The colour reaction may thus be used as a preliminary identification of the uronic acids."

The phenol-sulfuric acid color was run on a sample of alginic acid converted to its sodium salt. For comparison, the color reaction was run on Kelgin LV

(Kelco Corporation) which is the sodium salt of alginic acid containing 20% L-guluronic and 80% D-mannuronic acids (34). The absorbance of mannuronic and guluronic acid was obtained from the results of Haug and Larsen (33). The absorbance of the polyuronide containing only sodium anhydrouronate units may be compared to the absorbance of a monouronide because the molecular weight of a monouronic acid (194) is similar to the molecular weight of a sodium anhydrouronate unit (198).

The results of the phenol-sulfuric acid color test are seen in Fig. 2. From the position of the absorption line, this figure shows that the alginic acid from L. hyperborea stipes is approximately 75% guluronic acid and 25% mannuronic acid. These results agree with the results of paper chromatography.

The number average degree of polymerization was calculated from the intrinsic viscosity of the sodium alginate in acetate buffer at pH 5.50 (17).

Propionyl esters were determined quantitatively by an adaptation of the hydroxylamine procedure of McComb and McCready (35) who used it to determine the acetyl content of acetylated pectin. This method utilizes the reaction between esters and hydroxylamine to produce hydroxamic acids. The reaction of acetylated pectin with hydroxylamine is seen in Fig. 3. The acetohydroxamic acid (II) is determined quantitatively by its formation of a soluble red ferric complex that is determined colorimetrically from its absorbance at 520 nm. Pectin hydroxamic acid (I) forms an insoluble red ferric ion complex. The formation of the precipitate is a qualitative test for esterification of the uronic acid carboxyl.

Since propionated algin is chemically analogous to acetylated pectin, the reaction should proceed in a similar manner. Subsequent work has shown that the

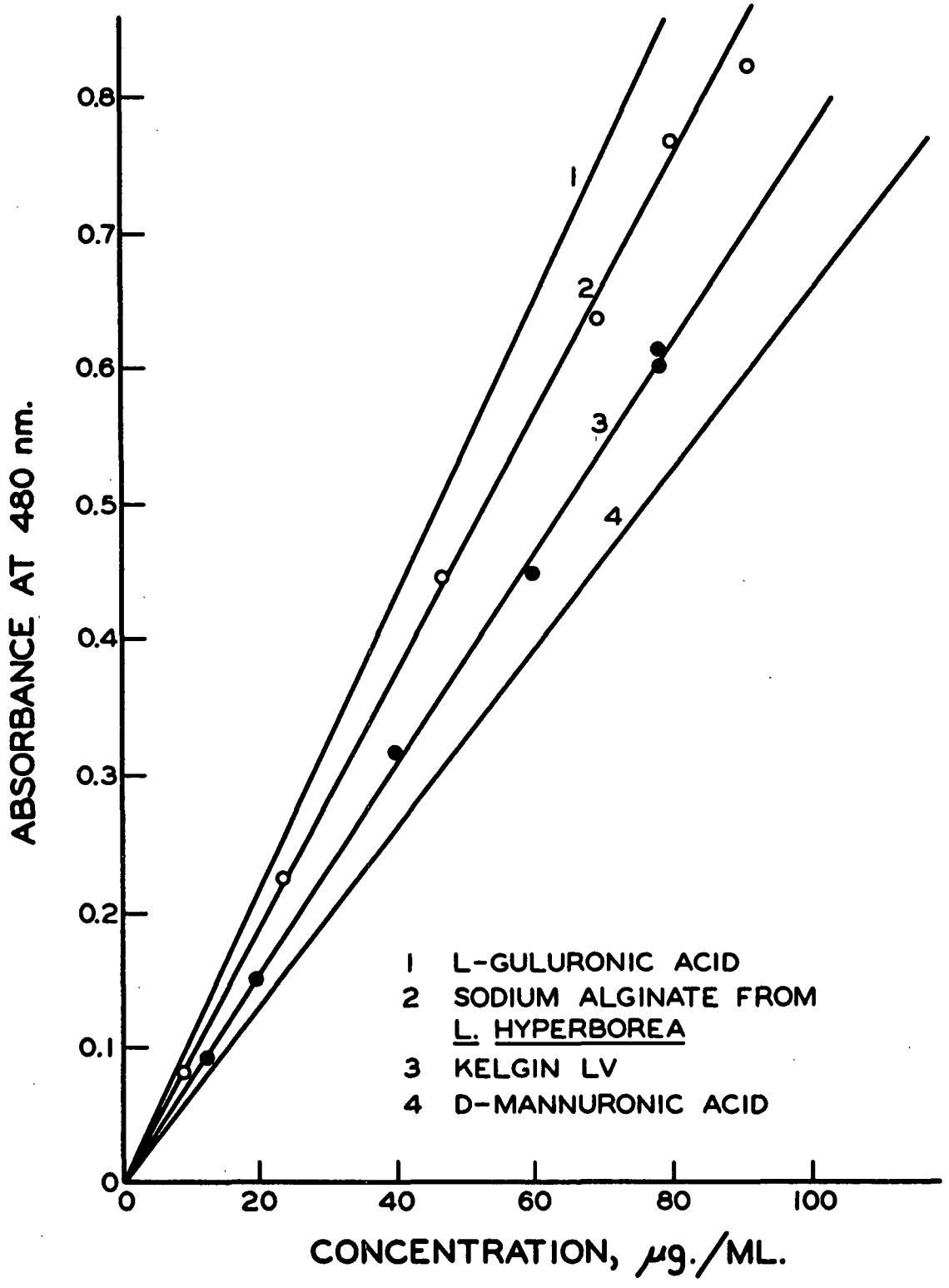


Figure 2. The Phenol-Sulfuric Acid Color Reaction for Different Alginic Acids

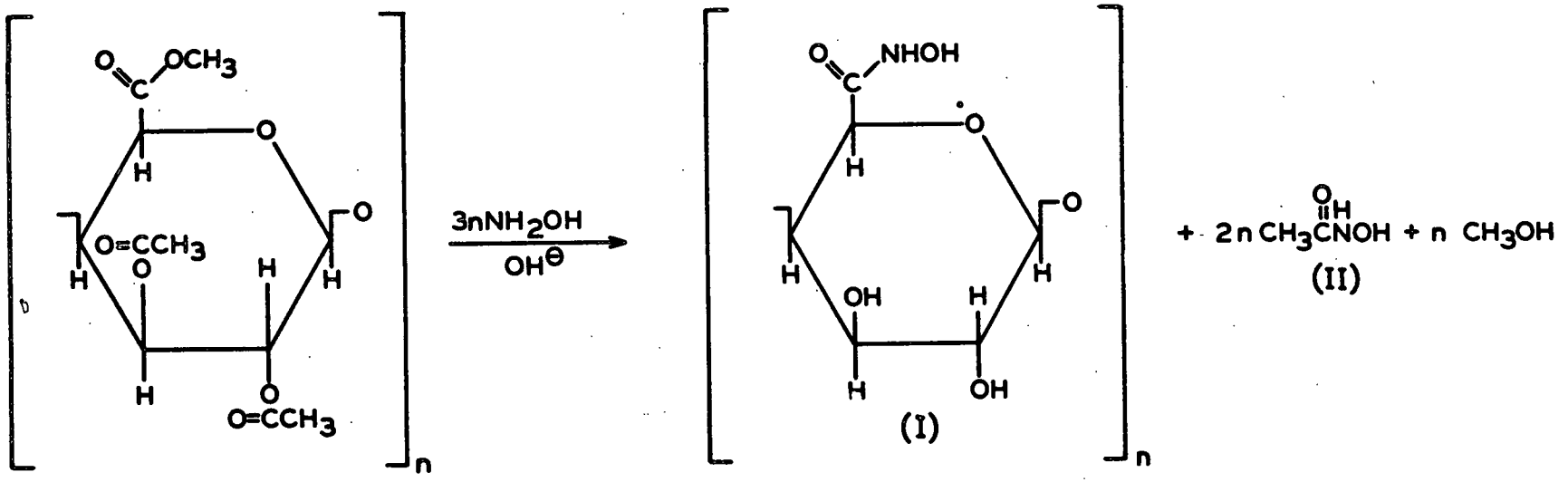


Figure 3. The Reaction of Acetylated Pectin with Hydroxylamine (35).

method does satisfy the requirements for a specific, sensitive, and rapid colorimetric procedure for a quantitative propionyl ester determination and a qualitative test for esterification of the uronic acid carboxyl.

The results from the analysis of alginic acid are seen in Table I.

TABLE I

ANALYSIS OF ALGINIC ACID

Yield from stipes (ovendry basis), %	23.2
Moisture, %	11.2[a]
Ash (corrected for carbonate), %	1.52[a]
Anhydrouronic acid (corrected for ash and moisture)	
By titration, %	96
By CO_2 evolution, %	94[a]
Esterification (uronic acid carboxyl and hydroxyl)	
By titration, %	0.0
By color test, %	0.00[a]
Number average degree of polymerization[b]	370
Sugar analysis[c]	75% guluronic, 25% mannuronic
Optical rotation[b]	$[\alpha]^{18°}_{Hg\ 546} = -130°$[d]

[a] The average of duplicate determinations.

[b] Determined on sodium alginate.

[c] No neutral sugars were detected.

[d] Concentration = 0.092 g./dl., in 0.1N NaOH.

The reason the alginic acid had less than 100% anhydrouronic acid is due to residual moisture present even in an ovendried alginic acid (31,36). The alginic acid is believed to be composed entirely of anhydrouronic acids since no neutral sugars were detected by paper chromatography.

The Donnan and Rose (17) relationship between intrinsic viscosity and molecular weight of sodium alginate has found wide acceptance among people working with alginic acid. In addition, it has been substantiated by other workers (37,38). Hence, the relationship is felt to be valid and accurate. The accuracy of the individual viscosity determinations for a linear extrapolation is seen in Fig. 4.

The ordinate axis in Fig. 4 is calculated from the equation

$$\eta_{sp/c} = (t-t_o)/t_o c$$

where η_{sp} is the specific viscosity and t and t_o are the efflux times of solution and solvent, respectively, and c is the concentration in g./dl.

PREPARATION OF DI-O-PROPIONYL ALGINIC ACID

A modification of the propionation procedure of Carson and Maclay (39) was employed in order to achieve complete propionation in a shorter time. The propionation is achieved by the addition of propionic anhydride to the polymer in pyridine – formamide solvent. The alginic acid, which had been recovered by freeze exchange through acetone and petroleum ether (b.p. 30-60°C.), was air dried to 11% moisture. This freeze-exchange alginic acid was never completely dried for two reasons. First, if the alginic acid remains moist, it disperses and reacts more readily (40). Second, if a small amount of water is present in the propionation reaction, the undesired dark resinous condensation product from the reaction of pyridine with propionic anhydride can be avoided (41).

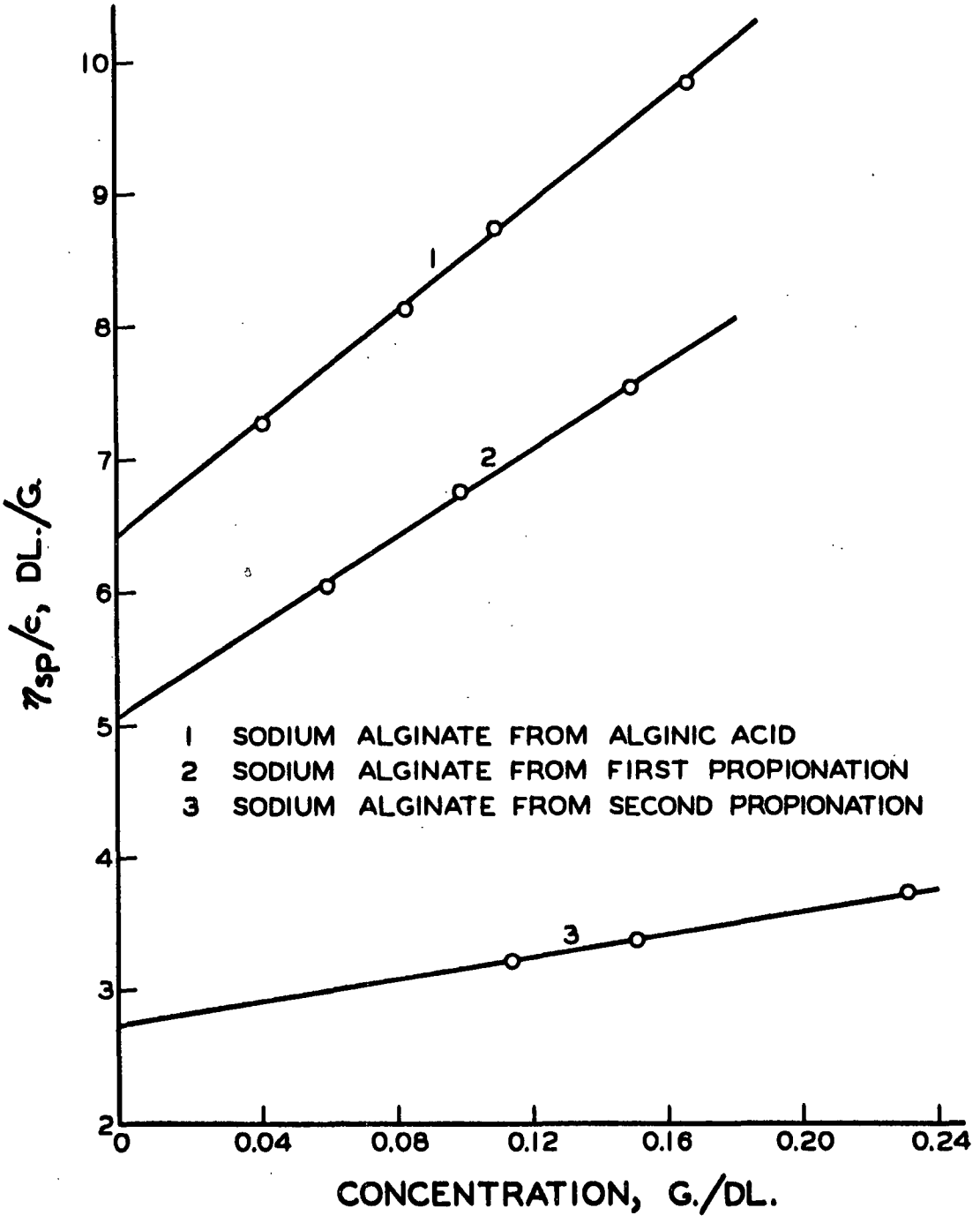


Figure 4. The Viscosity of Sodium Alginates in Acetate Buffer pH 5.50, at 30.0°C.

The results from the analysis of the propionated alginic acids after each propionation are seen in Table II. These analyses were conducted in the same manner as for the original alginic acid.

TABLE II

ANALYSIS OF PROPIONATED ALGINIC ACID

	Propionation Step	
	First	Second
Yield, % (corrected for propionyl and moisture)	93.2	85.6
Moisture, %	10.55	6.90
Ash, %		<0.1
Degree of propionyl substitution (per anhydrouronic acid)		
By titration	0.475	2.38
By color test	0.480	2.04
Number average degree of polymerization[a]	290	158
Uronic acid carboxyl esterification[b]	negative	negative
UV absorbance at 230 nm.[c] (c = 0.1 mg./ml.)	0.001	0.055

[a] Determined on water-soluble sodium alginate.

[b] This is a qualitative test.

[c] Determined for unsaturation in sodium alginate, discussed on p. 21.

One of the methods for the determination of propionyl ester was by titration with alkali. It is assumed that all of the alkali consumed upon titration to pH 7.5 reacts only with all the free carboxyls. Furthermore, it is assumed that alkali is consumed only by the saponification of propionyl esters when the pH is brought from 7.5 to 13.

The agreement between titration and color test for the analysis of propionyl esters is very good for the alginic acid from the first propionation step. This

agreement between the two methods shows that the assumptions for the titration analysis are valid when alginic acid has such a low degree of substitution that the polymer at titration to pH 7.5 is soluble. After the second propionation, the propionated alginic acid had such a high degree of substitution that the algin polymer was insoluble when titrated to pH 7.5. Hence, not all the uronic acid carboxyls were accessible to the alkali. When an excess of alkali was added to the polymer, in addition to all the esters reacting, all the remaining uronic acid carboxyl groups consumed alkali as the polymer went into solution. This made the esterification appear higher than is theoretically possible. In the hydroxylamine-ferric perchlorate color test, this problem of insolubility is not encountered so the color test will give valid results at all degrees of substitution (35).

The difference in the amount of propionyl substitution between the first and second propionation is due to a difference in solubility. In the first propionation, the alginic acid remained insoluble throughout the reaction, because the alginic acid was added to a pyridine – formamide solvent mixture. In the second propionation, the partially propionated alginic acid was added to formamide which dissolved the polymer. The solution was diluted with pyridine and reacted with propionic anhydride. The polymer remained in solution and all the remaining hydroxyls were substituted.

The number average degree of polymerization of each of the propionated alginic acids was calculated from the intrinsic viscosity of the ester-free sodium alginate. This sodium alginate was obtained by adding enough alkali to the propionated alginic acid to bring the pH to 11. The excess alkali and sodium propionate were removed by dialysis. The intrinsic viscosity of the sodium alginates was determined in acetate buffer at pH 5.50, the same as for the original alginic acid. The extrapolation of the individual viscosity determinations is seen in

Fig. 4, page 17. It is assumed in this procedure that the alkali treatment does not cause depolymerization of the propionated alginic acid. Whistler and BeMiller (42) have shown that alginic acid is more resistant to alkaline degradation than neutral polysaccharides. In addition, any alkaline degradation proceeding by the alkaline "peeling" reaction does not cause much lowering of the molecular weight. If the uronic acids are esterified, however, the alkaline depolymerization is rapid, very likely due to the β-alkoxy carbonyl mechanism (see Fig. 5), causing random cleavage.

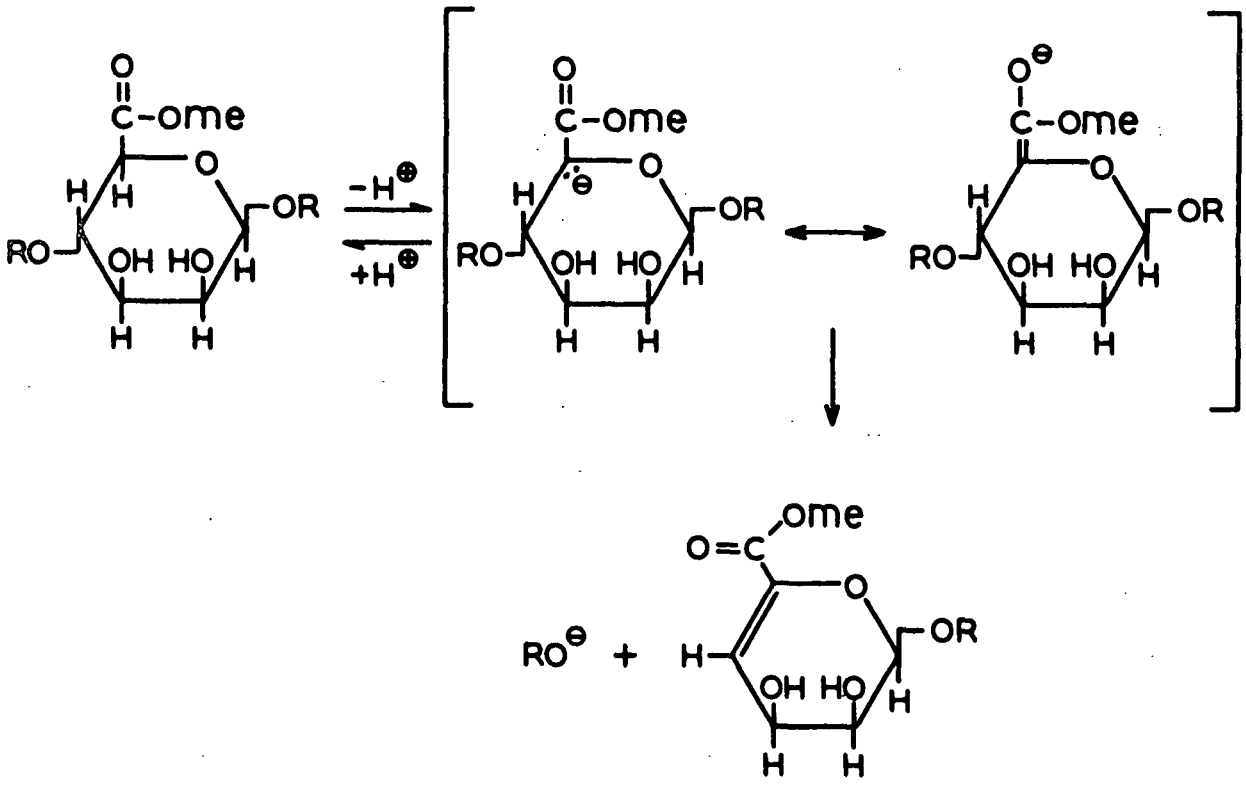


Figure 5. The Alkaline Depolymerization of Methyl Alginate by the β-Alkoxy Carbonyl Mechanism (Where R = the Remaining Portion of the Polysaccharide) (43)

Two different analyses have been made to show that the β-alkoxy carbonyl mechanism does not cause depolymerization. First, the qualitative color test (35) has failed to show that any of the uronic acid carboxyls are esterified. Second, the sodium alginates showed no peak absorbance at 230 nm. If the β-alkoxy carbonyl mechanism were occurring, it would cause 4-5 unsaturation. A uronic acid with 4-5 unsaturation is known to have an absorbance peak of 230 nm. (44-46). The small amount of UV absorbance observed is probably due to a trace of pyridine since a 1×10^{-7} molar solution of pyridine has an absorbance of 0.23 at 230 nm. with a peak absorbance at 260 nm. The sodium alginate derived from di-O-propionyl alginic acid had a peak absorbance even at 260 nm. which would be due to pyridine, and not 4-5 unsaturation.

Because of these analyses no chain scission is believed to occur. The intrinsic viscosity of the derived sodium alginate is believed to show the actual degree of polymerization of the propionated alginic acids.

PREPARATION OF METHYL DI-O-PROPIONYL ALGINATE

In order to obtain a reduced alginic acid using lithium borohydride, the uronic acid carboxyls should be esterified (4). However, the conditions described in the literature for the diazomethane methylation of polysaccharide all involve a heterogeneous reaction. The insolubility of the polysaccharide is believed responsible for the low degree of substitution that is obtained.

When the esterification reaction was conducted in tetrahydrofuran – diethyl ether solvent mixture di-O-propionyl alginic acid was highly swollen and yielded a polymer high in methoxyl. As a result, this procedure was used to methylate di-O-propionyl alginic acid. The polymer from this reaction had 89% of its uronic acids esterified determined by a TAPPI method (47), whereas methylation in diethyl ether (48) gave a polymer having only 74% of its uronic acid carboxyls esterified.

PREPARATION OF REDUCED ALGIN POLYSACCHARIDES

Four different reduced polysaccharides were prepared in the present study. A diagram of the origin of each of the polysaccharides is seen in Fig. 6.

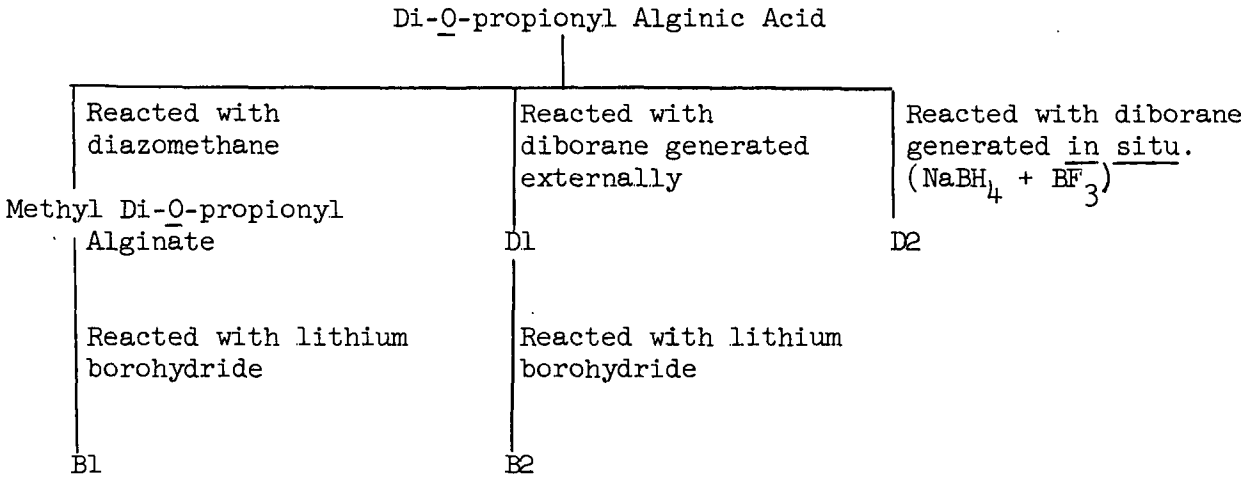


Figure 6. Reduction of Algin Polyuronides

PREPARATION WITH LITHIUM BOROHYDRIDE

In order to determine the relative reactivity of the functional groups of a soluble polysaccharide toward a Lewis base, methyl di-O-propionyl alginate was reduced with lithium borohydride in boiling tetrahydrofuran (4). This polymer had all of its C-2 and C-3 hydroxyls substituted with n-propionyl esters, 89% of the uronic acid carboxyls as a methyl ester, and 11% of its uronic acid carboxyls in the free acid form. Lithium borohydride was used in the ratio of 7 moles per mole of ester.

The reduced polysaccharide was designated B1 to show that it was reacted only with borohydride.

In the same manner, a portion of the polysaccharide which had been partially reduced with diborane (D1) was made to react with lithium borohydride. This

polysaccharide, D1, had only 1 out of 10 hydroxyl groups propionated, 68% of its hemiacetal end groups unreduced, and 11% of its uronic acid carboxyl groups in the free acid form. The other 89% of the uronic acid carboxyl groups had been reduced with diborane to primary alcohols. Because of the low propionyl ester content this polysaccharide was insoluble during lithium borohydride reduction. The percent reduction of the functional groups on this insoluble polysaccharide was compared to the percent reduction of the functional groups on the soluble polysaccharide, methyl di-O-propionyl alginate. In this way, it was shown how insolubility affected the percent reduction of the functional groups linked to a polymer.

The lithium borohydride reduction of D1 yielded a polysaccharide designated B2 to show that it was reduced by a two-step procedure with borohydride being the last step.

PREPARATION WITH DIBORANE GENERATED IN SITU

Smith and Stephan (3) applied the diborane reduction procedure to simple acidic carbohydrates and acetylated (or propionated) carbohydrate polymers. They showed that 82% of the uronic acid carboxyls of di-O-propionyl alginic acid were reduced when diborane was generated in situ. Because of their success in the reduction, their procedure was used for the present reduction.

Diborane was generated in situ by the addition of boron trifluoride etherate, a strong Lewis acid, to a solution of di-O-propionyl alginic acid and sodium borohydride, a Lewis base, in bis (2-methoxyethyl) ether ("diglyme"). The following reaction takes place (1):

$$3NaBH_4 + 4BF_3{:}O(C_2H_5)_2 \rightarrow 2B_2H_6 + 3NaBF_4 + 4(C_2H_5)O.$$

The dosage of sodium borohydride was 9.75 moles/carboxyl, equivalent to 6.5 moles of diborane/carboxyl. This is a considerable excess of diborane over the theoretical requirement of one-half mole according to the following reaction (1):

$$RCO_2H + BH_3 \rightarrow RCH_2OBO + H_2.$$

The polysaccharide from the reduction was reacted with water to convert the borate to an alcohol. The following reaction takes place (1):

$$RCH_2OBO + 2H_2O \rightarrow RCH_2OH + H_3BO_3.$$

Since the polysaccharide from the reduction now was contaminated with boric acid and $NaBF_4$, it was necessary to remove these salts before the polysaccharide could be analyzed. Removal of these salts was done under mild conditions.

After trying several recovery procedures, a sodium bicarbonate neutralization was found to give the highest yield of polysaccharide. In this procedure, all the acidic carboxyls are neutralized to their sodium salt by adding enough sodium bicarbonate to bring the pH to 7.4. The salts were removed by dialysis. Analysis of the water outside of the dialysis bags for carbohydrate by the phenol – sulfuric acid color test (33) showed that after 68 hours only 1% of the total carbohydrate had low enough molecular weight to diffuse through the dialysis membrane. In addition, the neutralization was not alkaline enough to cause saponification of the propionyl esters.

When the salts were removed simply by dialysis of the unneutralized polymer, only 10% of the polysaccharide could be recovered. Presumably, depolymerization occurred by autohydrolysis. Davidson and Standing (49) have shown that acidic oxycellulose, containing about 10% glucuronic acid, is inherently unstable when the uronic acid carboxyls are in the free acid form and some moisture is present.

In order to account for this phenomenon, they hypothesized that autohydrolysis occurs through hydrogen ions derived from the ionization of carboxyl groups. Since the polysaccharide from the reduction has some of the uronic acids unreduced, the same autohydrolysis could occur.

The resulting polysaccharide from the bicarbonate recovery was called D2 to show that it was reduced with diborane in the presence of two other reactive chemicals, $NaBH_4$ and BF_3.

Since reduction with diborane generated *in situ* is complicated by the presence of BF_3 and $NaBH_4$, the di-O-propionyl alginic acid was subjected to reduction with diborane generated externally (6). In this way, the only difference in the reduction would be the presence or absence of sodium borohydride and boron trifluoride in the polymer solution.

PREPARATION WITH DIBORANE EXTERNALLY GENERATED

Diborane was generated in an external flask by the addition of boron trifluoride to a solution of sodium borohydride in bis (2-ethoxyethyl) ether. The gaseous diborane was swept from the generation flask into the polymer solution with a stream of dry nitrogen (6). The same ratio of diborane to polysaccharide was maintained as for diborane generated *in situ*. In addition, the concentration of the polysaccharide in diglyme in the reaction flask was kept the same as for diborane generated *in situ*. The reduced polysaccharide was recovered by the same sodium bicarbonate procedure already described.

The resulting polysaccharide was called D1 to show that only one chemical, diborane, was used in the reduction.

RESULTS OF ANALYSIS OF REDUCED POLYSACCHARIDES

The polysaccharides from each of the four different reduction procedures were analyzed to see how much each of the three functional groups (uronic acid carboxyls, propionyl esters, and hemiacetal end group) was reduced and how much depolymerization had occurred.

In order to determine how much of the propionyl ester was reduced, analysis for the propionyl ester left on the polysaccharide was not sufficient. In addition to the propionyl ester undergoing reductive cleavage, a portion of the propionyl ester is reduced without cleavage to the ether. Both Hirst, et al. (5) and Ross and Thompson (6) have shown that esters on polysaccharides are reduced to a small extent to ethers. Hirst, et al. (5) have shown that di-O-propionyl alginic acid reduced with diborane generated in situ yielded a polysaccharide containing 5.2% n-propoxyl. Ross and Thompson (6) have shown that when acetylated 4-O-methylglucuronoxylan was reduced with diborane generated in situ, the apparent methoxyl content had increased to 4.84% whereas the original methoxyl content was 2.19%. By employing a gas chromatographic technique (8) for the separation of alkyl iodides Ross found a reduced polymer to contain both methoxyl and ethoxyl groups.

In the present study, in addition to the analysis of total n-propoxyl by a modified Zeisel determination, a portion of the n-propoxyl was found to be alkali labile. When reduced polysaccharide was heated to boiling (100°C.) in 1.82N caustic for 30 minutes, a portion of the total n-propoxyl came over in the distillate as n-propanol. The n-propanol was identified by its relative gas chromatographic mobility on two columns. In addition, the n-propanol was identified by conversion to n-propyl acetate. (See p. 66 for procedure.) The n-propyl acetate was identified by comparison of its gas chromatographic mobility to an authentic sample of n-propyl acetate.

By collecting the distillate from the alkaline saponification of the reduced polysaccharide for 30 minutes, a 98% recovery of all the alkali-labile n-propoxyl could be achieved. The amount of n-propanol in the distillate was quantitatively determined by the area under the peak on the gas chromatograph. The percent of n-propoxyl that came off a given polysaccharide with alkali was a highly reproducible value.

Several analyses have shown that the alkali-labile n-propoxyl arises from the alkaline cleavage of n-propyl ethers. A portion of two polysaccharides, D1 and D2, were deesterified under mild conditions (pH 8.0, room temperature) with hydroxylamine so that depolymerization did not occur. (This procedure is found on p. 69.) Even after deesterification, these polysaccharides had within experimental error the same total and alkali-labile n-propoxyl content as their respective parent polysaccharide. In addition, the lithium borohydride reduction of D1 did not change its total n-propoxyl content.

The results of the analysis for the functional groups and molecular weight of the reduced polysaccharides are shown in Table III.

In addition to the results presented in Table III, the polysaccharides were analyzed by qualitative paper chromatography. All these polysaccharides were hydrolyzed with acid (N H_2SO_4, 6 hr. at 95°C.). The acid hydrolyzates were analyzed for lactones and uronic acids by the method of Fisher and Dörfel (13) and the neutral sugars by the paper chromatographic procedure of Hirst, et al. (5).

The paper chromatographic analysis confirms the results presented in Table III. All the polysaccharides gave gulose and mannose as their major constituents. The gulose-to-mannose ratio appeared to be the same as the guluronic-to-mannuronic ratio found in the original alginic acid (3G:1M). All polysaccharides showed

TABLE III

ANALYSIS OF THE POLYSACCHARIDES OBTAINED FROM ALGINIC ACID BY DIFFERENT REDUCTION PROCEDURES

Reduction Procedure	Polymer	Functional Group: Anhydrouronic Acid, %[a]	n-Propionyl Ester, %[b]	Total Propoxyl, %	Alkali-Labile n-Propoxyl, %	Esterified Uronic Acids[b]	Degree of Polymerization, $\overline{DP}_N$[c]
Methyl di-O-propionyl alginate with $LiBH_4$	B1	6.3	0.00	0.00[d]	0.00	negative	103
D1 with $LiBH_4$	B2	10.4	0.00	3.9	0.25	negative	83
Di-O-propionyl alginic acid with B_2H_6	D1	11.0	7.6	3.5	2.3	negative	111
Di-O-propionyl alginic acid with $NaBH_4 + BF_3$	D2	16.3	3.2	7.5	2.7	negative	66

[a] Determined by CO_2 evolution.

[b] Determined by the hydroxylamine – ferric perchlorate color test.

[c] Determined by osmometry of the triacetate in 1,1,2 trichloroethane.

[d] Contained 0.40% methoxyl from diazomethane methylation of di-O-propionyl alginic acid.

uronic acids present. The uronic acids were present in sufficient quantity in all polysaccharides except B1 to be identified as guluronic and mannuronic acid from the mobility of their respective lactones and free acids. The uronic acid content of B1 was so low that when an attempt was made to resolve the uronic acids, the p-anisidine hydrochloric acid spray could not detect them. All polysaccharides except B1 gave an identical fast-moving spot believed due to n-propoxyl ethers of gulose and mannose.

The results of Table III become more meaningful if they are expressed in terms of percent reduction of the different functional groups. The methods for calculating the percent reduction of the functional groups is found on p. 44. The results from the calculations for percent reduction of all the functional groups is seen in Table IV.

TABLE IV

PERCENT REDUCTION OF THE FUNCTIONAL GROUPS ON ALGINIC ACID BY DIFFERENT REDUCTION PROCEDURES

	Reduced Polysaccharides			
	B1	B2	D1	D2
Reduction of uronic acid carboxyls, %	43	5.5	89.0	83.7
Reduction of esterified uronic acid carboxyls, %	100	--	--	--
Reductive cleavage of n-propionyl ester, %	100	<94[a]	74	74
Reduction of n-propionyl ester to n-propyl ether, %	0.0	0.0	8.4	18
Reduction of hemiacetal end group, %	100	94	32	64

[a]Not calculated but obtained by inference.

REDUCTION WITH LITHIUM BOROHYDRIDE

When lithium borohydride, a Lewis base, reacts with a propionated polyuronide, the esters undergo reductive cleavage only, the hemiacetal end groups are reduced to glycitols, and some of the uronic acid carboxyls are reduced to primary alcohols. The extent to which these three reactions proceed depends on the solubility of the polysaccharide.

Methyl di-O-propionyl alginate, initially having 89% of its uronic acid carboxyls esterified, was soluble in the reaction solvent. The resulting polysaccharide, B1, from the reduction had all its n-propionyl esters and methyl esters reductively cleaved. All the hemiacetal end groups were reduced to the alcohol, forming a glycitol. In addition, 43% of the uronic acid carboxyls were reduced to primary alcohols.

The polysaccharide from diborane reduction, D1, was insoluble at all times during subsequent lithium borohydride reduction yielding B2. This insolubility affected the extent of reduction of both the hemiacetal end group and the uronic acid carboxyl, but to different extents. The reduction of the hemiacetal end group was not affected as much as the reduction of the uronic acid carboxyl. Rees and Samuel (4) hypothesize that the reduction of the functional groups on a polysaccharide under heterogeneous conditions is incomplete because not all the molecules of the solid phase are accessible to the reagent. Since B2 had 94% of its hemiacetal end group reduced, an alternative explanation for incomplete and different extents of reduction of the functional groups is based upon activation energies. In studying the relative reactivity of soluble simple organic molecules to reduction with sodium borohydride, aldehydes were found to react more rapidly than carboxylic acids (2). By application of Arrhenius' law:

$$k = k_o e^{-E/RT}$$

where k is the rate of the reaction, k_o is the frequency factor, E is the activation energy of the reaction, R is the gas law constant, and T is the absolute temperature, the conclusion may be made that the activation energy for the reaction of an aldehyde is lower than the activation energy for a carboxylic acid. For an insoluble polysaccharide, the functional groups react more slowly because solvation energy (50) is added to the activation energy. The addition of a constant, called solvation energy, to each activation energy would increase the activation energy for each functional group. However, the activation energy for aldehydes would still be lower than the activation energy for carboxylic acids. As a result, aldehydes would react more rapidly than carboxylic acids under heterogeneous conditions. This conclusion is in agreement with experimental observations.

The reduction of esters was purposely omitted from this discussion because of the experimental difficulties in obtaining a true percent reduction of esters. In order to terminate the reduction reaction, the reaction was cooled and water was added to react with the excess lithium borohydride. The following reaction occurs (51):

$$LiBH_4 + 4H_2O \rightarrow H_3BO_3 + 4H_2 + LiOH.$$

The lithium hydroxide that is formed will saponify the esters, but this could not be avoided. If acid was added to the reaction before water in order to neutralize the alkali, the following reaction would liberate diborane (51):

$$LiBH_4 + HCl \rightarrow 1/2B_2H_6 + H_2 + LiCl.$$

The resulting diborane could react with the polysaccharide, making interpretation of reduction with lithium borohydride difficult.

In the reduction of methyl di-O-propionyl alginate, all the esterified uronic acid carboxyls were assumed to be reductively cleaved. This conclusion is based on two observations: (1) If a portion of the uronic acid carboxyl groups were still esterified at the end of the reaction, the subsequent alkaline recovery procedure would cause random chain cleavage by the β-alkoxy carbonyl mechanism shown on p. 20. This random cleavage would lower the molecular weight and introduce unreduced hemiacetal end groups. The analysis of the polysaccharide (B1) from the reduction has shown, however, that all the hemiacetal end groups are reduced and the polysaccharide has a high molecular weight ($\bar{DP}_N$ = 103). (2) More uronic acid carboxyl groups were reduced than were originally esterified. Since alkali metal borohydrides reduce esters more rapidly than acid carboxyl groups (2), the small amount of acid carboxyl groups remaining after reduction are probably the result of incomplete reduction of the less reactive acid carboxyl groups.

When the insoluble polysaccharide D1 was reduced with lithium borohydride, the n-propionyl esters probably were not 100% reductively cleaved. Hemiacetal (aldehyde) is supposed to react more rapidly than esters toward alkali metal borohydrides (2). Since only 94% of the hemiacetal end groups were reduced under heterogeneous conditions, an even lower percentage of the n-propionyl ester would be expected to react.

REDUCTION WITH DIBORANE

Reduction by lithium borohydride, a Lewis base, appears to involve transfer of the hydride ion from the anion to an electron-deficient center of the functional group (51). On the other hand, diborane is a strong Lewis acid. It would be expected to react by a preferred electrophilic attack on the centers of highest electron densities (1).

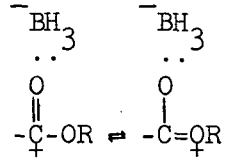
In this study a comparison of the percent reduction of the functional groups on algin polymers shows that there is indeed a marked difference in the order of reactivity. The relative reactivity of the functional groups of the soluble di-O-propionyl alginic acid toward diborane is in the following order: uronic acid carboxyls > reductive cleavage of n-propionyl esters > reduction of hemiacetal end group. In contrast, toward lithium borohydride reduction the order of reactivity is reversed from the reactivity of diborane: hemiacetal end group > reductive cleavage of n-propionyl ester > uronic acid carboxyl.

The fact that the uronic acid is the most reactive functional group toward diborane and the least reactive toward lithium borohydride is explained on the basis of the electrophilicity of the reductant. A uronic acid carboxyl has a center of high electron density so it would attract a strong electrophile such as diborane and repel a nucleophile such as the borohydride anion. There are two reasons, however, why diborane does not completely reduce all the uronic acid carboxyls: (1) Diborane is simultaneously removing n-propionyl esters by reductive cleavage, causing the polysaccharide to precipitate. From the discussion of the effect of solubility on the reduction with lithium borohydride, it may be inferred that insolubilization would slow the reduction of uronic acid carboxyls to diborane. (2) By the time the polysaccharide precipitated, all the diborane had been added. There is a considerable excess of diborane at this point (6.5 moles of diborane/carboxyl). The presence of this large excess of the strong Lewis acid probably suppresses the ionization of the uronic acid carboxyl. An unionized carboxyl would not attract diborane as readily as an ionized carboxylic acid.

The difference between the reactivity of the hemiacetal end group toward lithium borohydride and diborane is due to the difference in the acidity of the

reaction mixture. In the hemiacetal form, the end of the polysaccharide would not be reduced. However, the hemiacetal form of the reducing sugar is a function of pH; the quantity of hemiacetal decreases and the quantity of aldehyde increases rapidly as pH increases (53). Both diborane and lithium borohydride rapidly reduce aldehydes (2). Since a solution of lithium borohydride would be more alkaline than a solution of diborane, the concentration of the aldehyde form of the reducing end group would be higher in the lithium borohydride solution than in a diborane solution. As a result, the reducing end of the polysaccharide is reduced more with lithium borohydride than with diborane.

In the comparison of the reduction of soluble polyuronides both diborane and lithium borohydride reduce n-propionyl esters with cleavage, but lithium borohydride reduces esters more readily. This result is explained by the difference in the way borohydride and diborane are attracted to an ester. Diborane is attracted to the electron-rich center of the ester, the oxygen. This means that the addition of diborane to the oxygen atom of the carbonyl group must compete with the addition to the oxygen atom of the alkoxy group. In addition, the transfer of hydride from boron will presumably be hindered by the stabilization provided by the carbonyl group by resonance with the oxygen atom of the alkoxy group (2).



The reduction of the ester with borohydride is not beset by either of these problems since reduction involves transfer of the hydride ion from the anion to the electron-deficient center of the functional group (52), in this instance to the carbonyl carbon.

For the first time, an ester has been shown to be reduced with diborane without cleavage to an ether. Only Ross and Thompson (6) have reduced an acidic polysaccharide containing alkyl esters (fully acetylated 4-O-methylglucuronoxylan) with diborane generated externally, and analyzed the reduced polysaccharide for ethers. He found that a 20% drop in methoxyl occurred during the reduction and recovery procedure. He states, "Although this point was not investigated, it is probably due to loss of short chain polymers." From the present investigation an alternative explanation may be made. In this study, the first time alginic acid was isolated, it was dried by solvent exchange through ethanol and petroleum ether by the same procedure as Ross employed. The resulting alginic acid was reacted with propionic anhydride in a manner analogous to the way Ross reacted his polysaccharide with acetic anhydride. The propionated alginic acid, recovered by Ross's solvent-exchange procedure, gave a positive test for esterification of the uronic acid carboxyls (35) and contained ethanol which could be removed only with alkali. The ethanol was analyzed in the alkaline distillate by the conventional gas chromatographic procedure. When this propionated alginic acid was reduced with diborane generated externally and deesterified by Ross's procedure, the reduced alginic acid was so extensively depolymerized that 90% of the reduced alginic acid passed through the dialysis membrane. The 10% that was left showed a UV absorption peak at 230 nm. From these results, it was concluded that the alginic acid carboxyls became esterified with adsorbed ethanol and the subsequent alkali treatment caused depolymerization by the β-alkoxy carbonyl reaction shown on p. 20. If Ross also had esterification of his 4-O-methylglucuronic acid carboxyl, the subsequent alkali treatment would remove the 4-O-methyl group by the β-elimination reaction. This also explains why Ross obtained a figure of 90% reduction of the uronic acid carboxyls by CO_2 evolution but could not detect 4-O-methylglucuronic acid in his reduced polysaccharide.

This explains the drop in methoxyl groups Ross experienced, but it does not explain why Ross failed to observe reduction of acetyl to ethoxy. Ross probably got a much lower percent reduction of ester to ethers because he had only one fourth the concentration of diborane for the reduction of his polysaccharide than was used in the present reduction of di-O-propionyl alginic acid.

The reduction of n-propionyl esters to n-propyl ethers with an excess of diborane may be explained by an adaptation of the reaction hypothesized by Pettit and Jasturi (54) to account for the reduction of esters to ethers with diborane and boron trifluoride (Fig. 7). Their reaction is the same as the one presented except they had BF_3 added initially.

When di-O-propionyl alginic acid is reduced with diborane generated in situ, the reduction needs to be explained in terms of reaction with three chemicals: diborane, sodium borohydride, and boron trifluoride. The di-O-propionyl alginic acid was added to a solution of sodium borohydride in diglyme. Diborane was generated in situ by the slow addition of boron trifluoride to the solution. Thus, during the course of the reaction, the solution gradually became acidic. With the addition of boron trifluoride, the sodium borohydride concentration was lessened and the diborane concentration was made greater. As the sodium borohydride concentration became less, the added boron trifluoride was reacting less slowly to generate the diborane.

With this mental picture in mind and applying what is known as the reduction of a polysaccharide with an alkali metal borohydride and diborane, a course for the reaction of each of the functional groups can be presented.

The initially alkaline sodium borohydride solution would cause more of the hemiacetal end group to shift to the aldehyde form. This aldehyde group

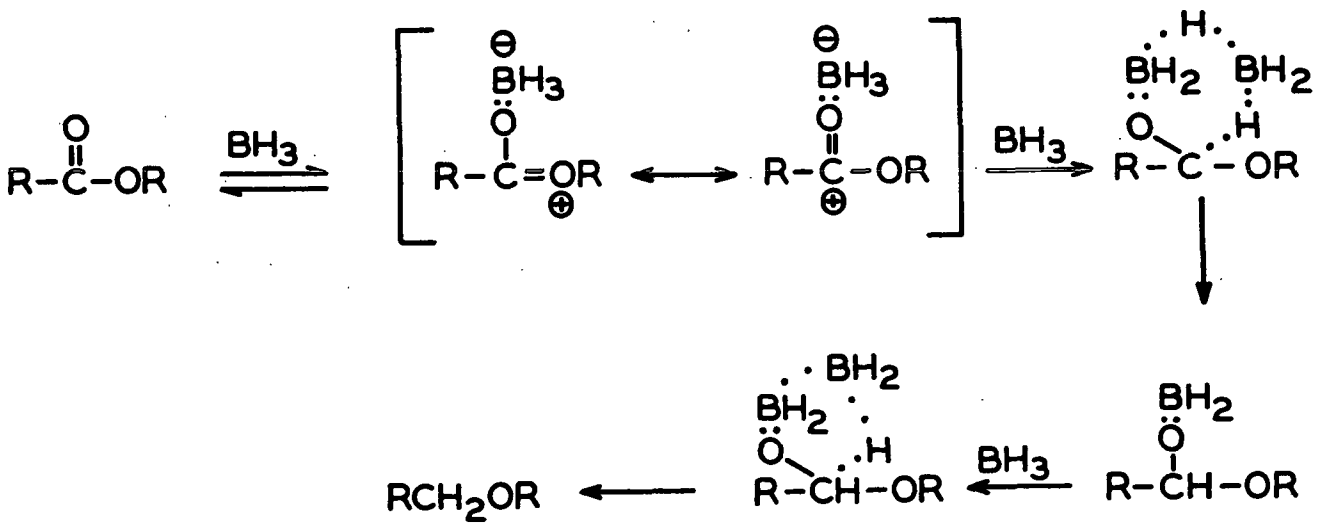


Figure 7. The Reduction of Esters to Ethers with an Excess of Diborane. [Adapted from Pettit and Kausturi (54)]

would be reduced with either sodium borohydride or diborane (2). As the reaction shifted to the acidic side, the unreacted aldehyde end group would revert back to its hemiacetal form and not be reduced further. This explanation accounts for the 64% reduction of the hemiacetal end group. This figure is intermediate to the percent reduction of the hemiacetal end group of B1 and D1.

The initial sodium borohydride solution is known to attack esters very slowly (55), so it probably does not affect the percent reductive cleavage of esters. The esters undergo reductive cleavage only with diborane. Hence, when diborane is either generated in situ or externally, the n-propionyl esters undergo the same percent reductive cleavage. This result may also be due to the fact that the n-propionyl esters impart solubility to the polymer. Therefore, when 74% of the esters are reductively cleaved, the polymer precipitates and the esters are no longer reduced.

The reaction shown on p. 37 can be used to explain why more n-propionyl esters are reduced to n-propyl ethers with diborane generated in situ than with diborane externally generated. When nearly all the boron trifluoride has been added, the sodium borohydride concentration had been reduced considerably, and boron trifluoride could be present to catalyze the reaction. Boron trifluoride is known to form stable coordination compounds with carbonyl oxygens (56). Hence, if the first step in the reaction shown on p. 37 were the rate-determining step, the addition of boron trifluoride would catalyze the reduction of esters to ethers.

The percent reduction of the uronic acid carboxyl was lower for D2 than for D1 when diborane was generated in situ for possibly two reasons: (1) Since boron trifluoride catalyzes the reduction of n-propionyl esters to ethers, it could also catalyze the reductive cleavage of n-propionyl esters. This increased

rate of reductive cleavage of the n-propionyl ester would cause the algin polymer to precipitate sooner and thereby lower the reactivity of the carboxyl group. (2) There is sodium present in the reaction when diborane is generated in situ. This sodium could form a sodium carboxylate which is not reduced with diborane.

THE EFFECT OF REDUCTION ON MOLECULAR WEIGHT

The number average degree of polymerization (DP) was determined on the reduced polysaccharides from the osmotic pressure of their triacetate derivative in 1,1,2-trichloroethane. The calculated DP's are found in Table III, p. 28.

The osmometer gave accurate and reproducible results which gave a linear extrapolation to zero concentration. A plot of the extrapolation of osmotic pressure, π, divided by concentration c, versus concentration for the four polysaccharides is seen in Fig. 8. The points for the extrapolation of B2 have more scatter than the other points because of the trouble with the temperature control on the osmometer. The points for the other polysaccharides were obtained with the osmometer, solvents, and solutions conditioned to ambient temperature with the temperature control off.

Although the osmometer gives accurate and reproducible results, the preparation of a derivative causes some inaccuracy. The acetylation procedure (acetic anhydride added to the polymer in pyridine – formamide) may cause some depolymerization of the polysaccharide. Since the uronic acid content would exert the biggest influence on the rate of acid hydrolysis, the work of Zitko and Bishop (57) is pertinent. They studied the effect of the uronic acid content on the rate of acid hydrolysis of partially reduced pectic acids. They showed that the higher the uronic acid content, the lower was the rate of acid hydrolysis. However, in the present study, the polysaccharide with the higher uronic acid content had the lower DP. The difference in DP must therefore be due to the different reduction procedures.

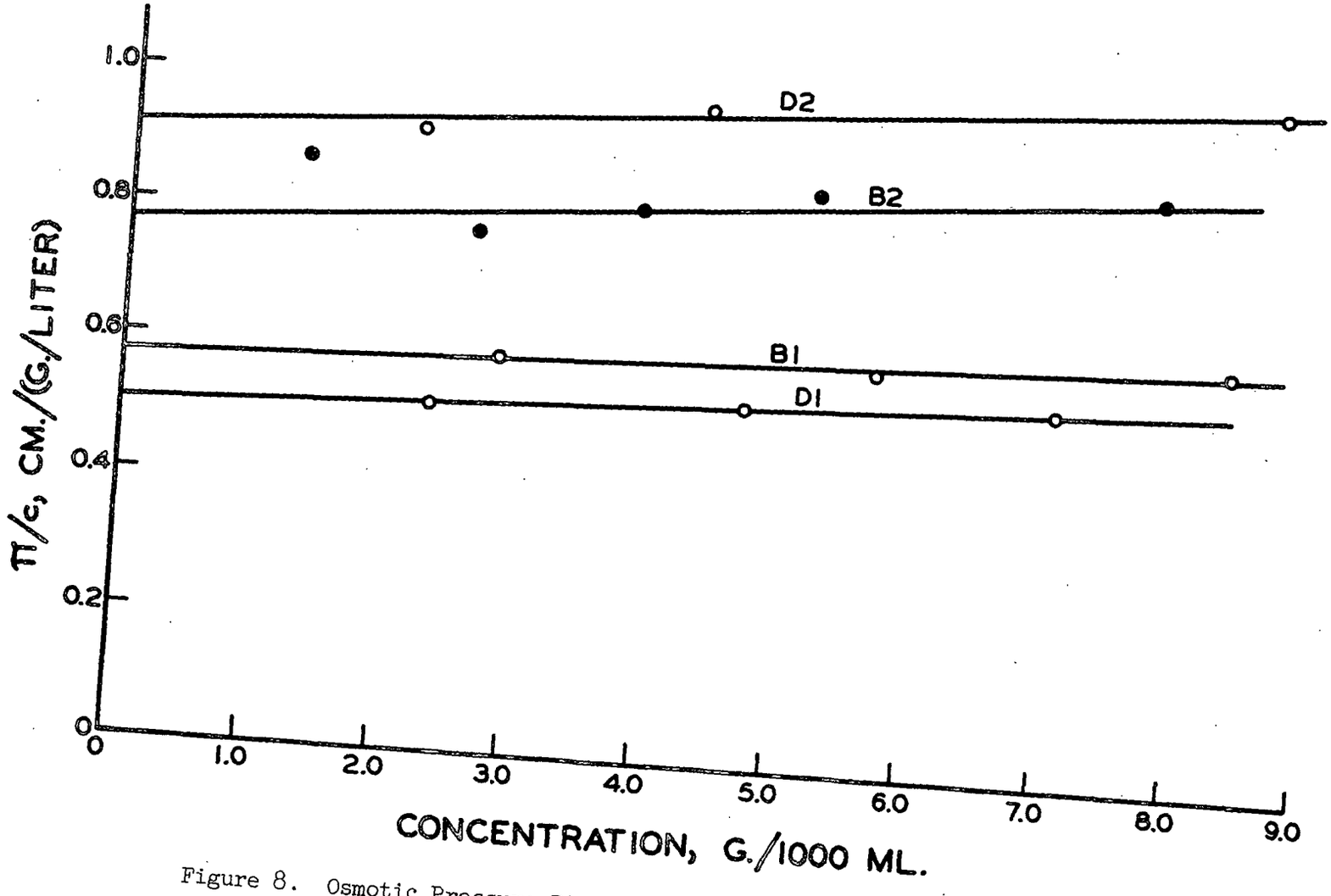


Figure 8. Osmotic Pressure Divided by Concentration *Versus* Concentration

Additional inaccuracy is introduced in DP determinations because the acetylated polysaccharide did not completely dissolve in 1,1,2-trichloroethane.

The solutions were filtered through a coarse sintered-glass filter before being injected in the osmometer. From a visual inspection of the filter, an estimated 5% of the highest molecular weight polymers, B1 and D1, was insoluble in 1,1,2-trichloroethane with lesser amounts of insoluble material in the lower molecular weight polymers, B2 and D2. Since the polysaccharides were found to be completely acetylated (35), the insoluble fraction cannot be due to a portion of the polysaccharide being incompletely acetylated. Instead, the insoluble fraction is probably a portion of the polysaccharide having a higher molecular weight. Because a small amount of high molecular weight polymer would exert a very low osmotic pressure, the osmotic pressure was determined on the soluble portion, but the concentration was not adjusted for the loss occurring during filtration.

The di-O-propionyl alginic acid before reduction had a number average degree of polymerization (DP) of 158. When this polymer was reduced with diborane, the DP dropped to 111. The lithium borohydride reduction of methyl di-O-propionyl alginate reduced its DP from 158 to 103. The difference in DP between these two reduced polymers probably is not significant because of the errors already discussed.

The polysaccharide from the two-step reduction, B2, had a DP of 83. The DP for B2 is lower than for D1 and D2 because of the additive effect of the two-step reduction.

The polysaccharide reduced with diborane generated in situ, D2, has the highest uronic acid content and the lowest DP of 66. The presence of the strong Lewis acid, boron trifluoride, is probably the primary cause for this lower DP.

Although boron trifluoride has not been used for the depolymerization of polysaccharides, Bonner, et al. (58) have used boron trichloride and boron tribromide to degrade cellulose acetate to glucose in 16 hours at room temperature. Hence, any boron trifluoride present may rapidly cleave glycosidic bonds. The degree of polymerization is probably greatly dependent on how fast and how much boron trifluoride is added to generate diborane in situ. This could explain why Zitko and Bishop (57) reported that extensive degradation occurred when they tried to reduce di-O-propionyl pectic acid with diborane generated in situ. The present reduction with diborane was successful because boron trifluoride was added slowly with vigorous stirring and an equivalent amount of sodium borohydride was present to react with all the boron trifluoride.

RECOMMENDED REDUCTION PROCEDURES

In conclusion, the lithium borohydride reduction procedure of Rees and Samuel is recommended for the reduction of acidic polysaccharides. This procedure was found to reduce completely the esterified uronic acid carboxyl groups to primary alcohols, to cleave reductively all the n-propionyl ester groups, and to reduce all the hemiacetal end groups to alditols. The polysaccharide from the reduction had no n-propyl ethers present. The procedure, however, did lower the number average degree of polymerization from 158 to 103. Because of its many favorable factors, the lithium borohydride reduction procedure is recommended when the reduction of the hemiacetal end group is unimportant.

The gaseous diborane reduction procedure of Ross and Thompson was conducted on di-O-propionyl alginic acid. Their procedure was found to reduce 89% of the uronic acid carboxyl groups to primary alcohols, 74% of the n-propionyl ester groups with cleavage to two alcohols, 8% of the n-propionyl ester groups without cleavage to the n-propyl ether groups, 32% of the hemiacetal end groups

to alditol end groups, and the DP dropped from 158 to 111. Since diborane is neither a selective reductant nor active enough to achieve complete reduction of the functional groups, its use is not recommended. This reduction procedure should be used only where reduction of the hemiacetal end group is not desired, such as the reduction of an aldobiuronic acid to a reducing disaccharide.

The reduction of di-O-propionyl alginic acid with diborane generated in situ offers no advantages over the reduction procedure of Ross and Thompson. Reduction with diborane generated in situ yielded a polysaccharide containing more n-propyl ethers and the lowest DP of 66. In addition, fewer uronic acid carboxyl groups were reduced and more of the hemiacetal end groups were reduced.

CALCULATION OF PERCENT REDUCTION OF FUNCTIONAL GROUPS

The percent reduction of the anhydrouronic acid carboxyls is based upon the amount of anhydrouronic acid carboxyl in the original acidic polysaccharide. In the case of polymers reduced with diborane, the di-O-propionyl alginic acid was considered to be composed entirely of anhydrouronic acid in the free acid form. Before reduction with lithium borohydride, each of the algin polymers had only 11% of its anhydrosugar units as an anhydrouronic acid in the free acid form. The percent reduction of the uronic acid carboxyl group is calculated from the equation:

$$\text{Reduction of uronic acid carboxyls, \%} = \frac{a_o - a}{a_o} \times 100.$$

In this equation, a_o is the percentage of anhydrouronic acid units before reduction and a is the percentage of anhydrouronic acid units after reduction.

Since only B1 had the uronic acid carboxyls esterified before reduction, it is the only polysaccharide included under the heading of percent reduction of esterified uronic acid carboxyls. After reduction, B1 had more uronic acid carboxyls reduced than were originally esterified. The conclusion was made, therefore, that 100% of the esterified uronic acid carboxyls had undergone reductive cleavage.

The percent reduction for the propionyl esters with and without cleavage was calculated by the following equations:

$$\text{Reductive cleavage of propionyl esters, \%} = \frac{40 - [x + (57/59)b]}{40} \times 100;$$

$$\text{Reduction of propionyl ester to n-propyl ether, \%} = \frac{(57/59)b}{40} \times 100.$$

In these equations x equals the percentage of n-propionyl group and b is the percent total n-propoxyl remaining on the polysaccharide after reduction. The ratio 57/59 is the ratio of the molecular weight of n-propionyl group to n-propyl group*. The number 40 is the percent n-propionyl group before reduction on di-O-propionyl alginic acid. These equations are valid for the polysaccharides B1, D1, and D2. In the case of B2, the original polysaccharide had only 7.6% n-propionyl ester before reduction. After reduction, the polysaccharide had no n-propionyl esters. Unavoidably, however, the propionyl ester may have also been removed by alkaline saponification during recovery. This saponification has already been discussed on p. 31. B2 before reduction also had 3.5% n-propoxyl. After reduction, the n-propoxyl content was 3.9%. The apparent increase in n-propoxyl is not real. Instead, the apparent increase is due to the 3.5% figure not being corrected for n-propionyl ester and the error in n-propoxyl determination may be ± 0.1% n-propoxyl.

The percentage of unreduced hemiacetal end group is obtained by making use of the difference in reactivity between reduced and unreduced sugars toward boiling alkali. Numerous investigators (6, 11, 59-61) have shown that a 2-O-bond is broken in alkali at 100°C. when it is attached to a terminal reducing sugar. The 2-O-substituent is stable to boiling alkali when it is linked to a glycosidic unit which has the C-1 position blocked so it cannot form a carbonyl group. Two examples where this stability has been demonstrated are: (a) when the one position is attached by a glycosidic linkage to another sugar, i.e., a nonterminal glycosidic unit (6, 11, 60) and (b) when the 2-O-substituent is attached to a terminal sugar which has been reduced to a glycitol (11).

*This ratio is the result of the way analyses are reported. The n-propionyl group does not include the sugar oxygen whereas the n-propoxyl group includes the sugar oxygen in its molecular weight.

Whistler and BeMiller (43) report that 3-O-substituents on terminal reducing sugars undergo alkaline cleavage by a β-alkoxy carbonyl elimination even at room temperature. This reaction is inoperative if the C-1 position is blocked so it cannot form a carbonyl group (43).

This means that reduction of the hemiacetal end of the polysaccharide molecule to form a glycitol should make the polysaccharide stable to alkali at 100°C. and the 2-O- and 3-O-n-propoxyl groups should remain intact. In contrast, a polysaccharide molecule having a hemiacetal end group should be degraded when subjected to the alkaline "peeling" reaction at 100°C. and all the 2-O- and 3-O-propoxyl groups should be labile. The percentage of the total n-propoxyl that is labile should be the percentage of the total end group that is in the hemiacetal (aldehyde) form.

The percent reduction of the hemiacetal end group is calculated from the following equation:

$$\text{Reduction of the hemiacetal end group, \%} = \frac{b - c}{b} \times 100.$$

In this equation, b is the total percent n-propoxyl and c is the percent alkali-labile n-propoxyl. The assumptions for this equation to be valid are the following:

1. Polysaccharide molecules containing either a reduced or an unreduced end group have the same average total n-propoxyl content.
2. The amount of n-propoxyl group blocking the reducing end of the polysaccharide molecule is negligible.
3. The polysaccharides containing a hemiacetal end group are completely degraded at 100°C. in 30 minutes.
4. The polysaccharides are not depolymerized by random chain cleavage.
5. None of the remaining uronic acid carboxyls are esterified.

The first two assumptions need no further elaboration. To check the validity of the third assumption, the distillate of D1 was collected as 1-ml. samples every 15 minutes and analyzed for n-propanol. The results showed 73.5% of the n-propanol was in the first 1 ml., 24.4% in the second ml., and 2.1% in the third ml. Thus, the reaction was 98% complete in thirty minutes.

The fourth assumption is supported by a survey of the literature which provided much evidence that random cleavage does not occur for a polysaccharide in 100°C. alkali. Brooks (62) has done a kinetic study of the alkaline cleavage of the glycosidic bond of methyl β-D-glucopyranoside. He concluded that since the activation energy for random cleavage is much greater than for the "peeling" reaction, it follows that at low temperatures, e.g., 100°C., the "peeling" reaction is the controlling reaction. This is in agreement with the observation that the degree of polymerization of cellulose in an alkaline medium at 100°C. is not reduced appreciably though considerable loss in polysaccharide is incurred (63-65). This type of degradation is characteristic of one proceeding primarily via the "peeling" reaction.

Numerous investigators have shown that reduction of the hemiacetal end of the polysaccharide to a glycitol stabilizes it against degradation in boiling alkali (11, 66-68). This stabilization would occur only if random cleavage of the polysaccharide is negligible since random cleavage would be unaffected by reduction of the hemiacetal end of the molecule.

It is realized that oxygen greatly promotes random cleavage (62), so the conditions for the distillation were designed to reduce oxygen to a minimum. The polymer-and-water slurry were deaerated by boiling for 30 minutes prior to the addition of alkali. The sodium hydroxide solution was prepared with freshly distilled water and stored in a stoppered flask. In addition, distillation from

the alkaline solution at atmospheric pressure should have removed the last traces of oxygen.

If the uronic acid carboxyls were esterified, alkali would rapidly cause random chain cleavage in a 1→4 linked polysaccharide by the β-alkoxy carbonyl mechanism shown on p. 20 (43). The qualitative hydroxylamine – ferric perchlorate color test (35) has shown, however, that none of the uronic acid carboxyls are esterified. Hence, the fifth assumption should be valid.

The validity of all five assumptions may be checked by analyzing for alkali consumption under conditions approximating the alkaline distillation conditions. In the study of the alkaline degradation of oligosaccharides, it has been shown that every equivalent of anhydrosugar consumes 1.2 equivalents of alkali. The oligosaccharides may be 1→2 linked (59), 1→3 linked (69), or 1→4 linked containing a 2-O-substituent (61). It makes no difference whether the oligosaccharides are reacted with calcium hydroxide at room temperature (69, 70), with sodium hydroxide at room temperature (59), or with sodium hydroxide at 100°C. (61); the limiting value for alkali consumption is 1.2 equivalents of alkali consumed per anhydrosugar unit.

When alkali reacts with a polysaccharide, the terminal reducing sugar undergoes complex rearrangements to form saccharinic acids. In 1→4 linked polysaccharides, the usual reaction product is an isosaccharinic group. The "peeling" reaction to form the isosaccharinate is seen in Fig. 9.

The formation of an isosaccharinate group from an anhydrosugar unit would imply that one equivalent of anhydrosugar unit would neutralize (consume) one equivalent of alkali. However, it has been shown that in addition to isosaccharinic acid there are formed, by fragmentation, acids of lower molecular

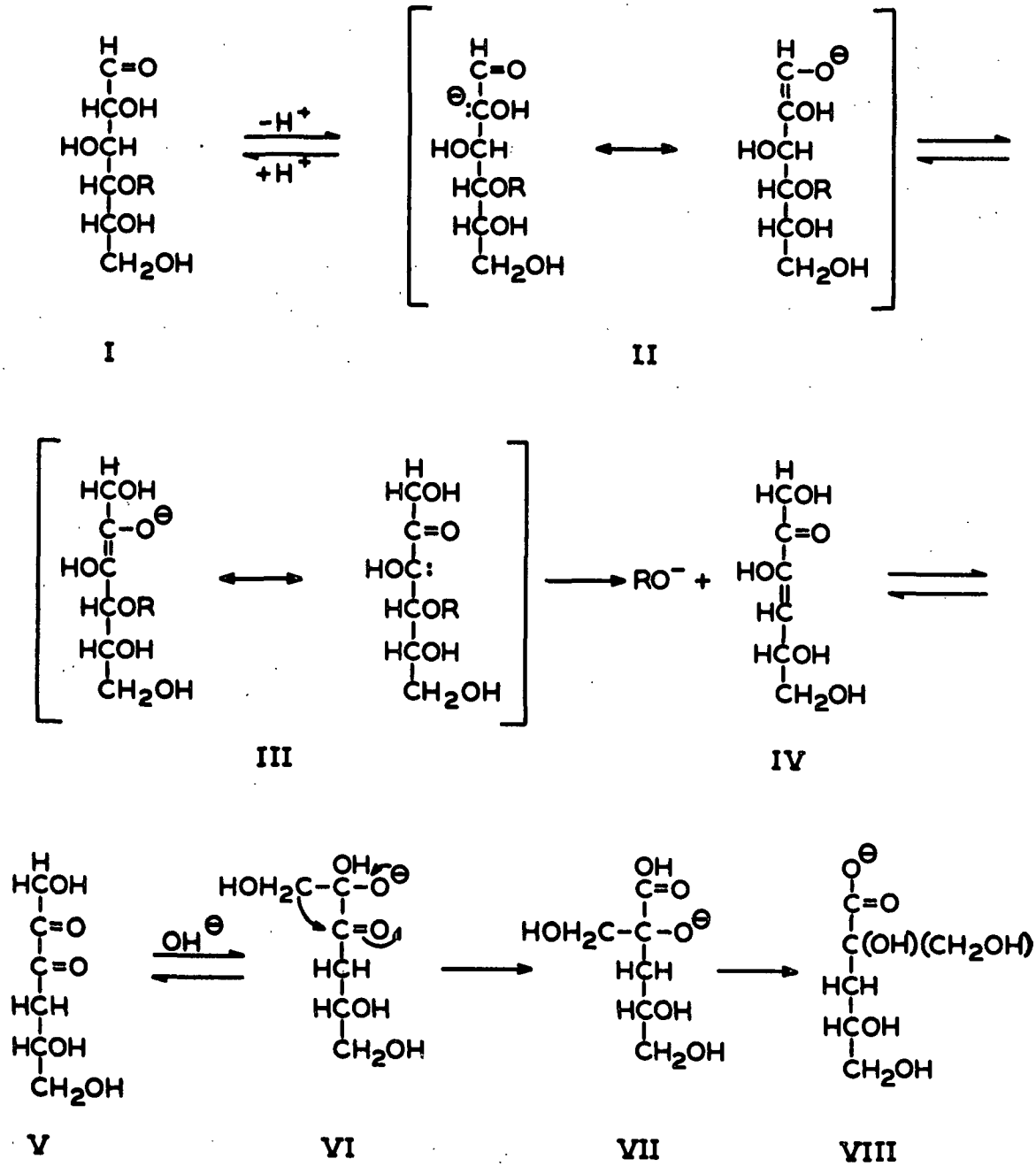


Figure 9. The Alkaline Degradation of a 1-4, Linked Polysaccharide by the "Peeling" Reaction (43)

weight such as formic, acetic, lactic (predominantly), and glycollic acids (61). The formation of these lower molecular weight acids accounts for the extra 0.2 equivalent of alkali consumed.

Since the end of the polysaccharide has to be in the hemiacetal form for the "peeling" reaction to commence, there should be a linear relationship between the percentage of end group reduced and the milliequivalents of alkali consumed. The alkali consumption was corrected for propionyl ester, and the results are seen in Fig. 10. The line represents the theoretical alkali consumption and the circle points are experimentally determined values. The excellent agreement provides evidence that the five assumptions are correct and that the method for calculating percentage of unreduced end group is valid and accurate.

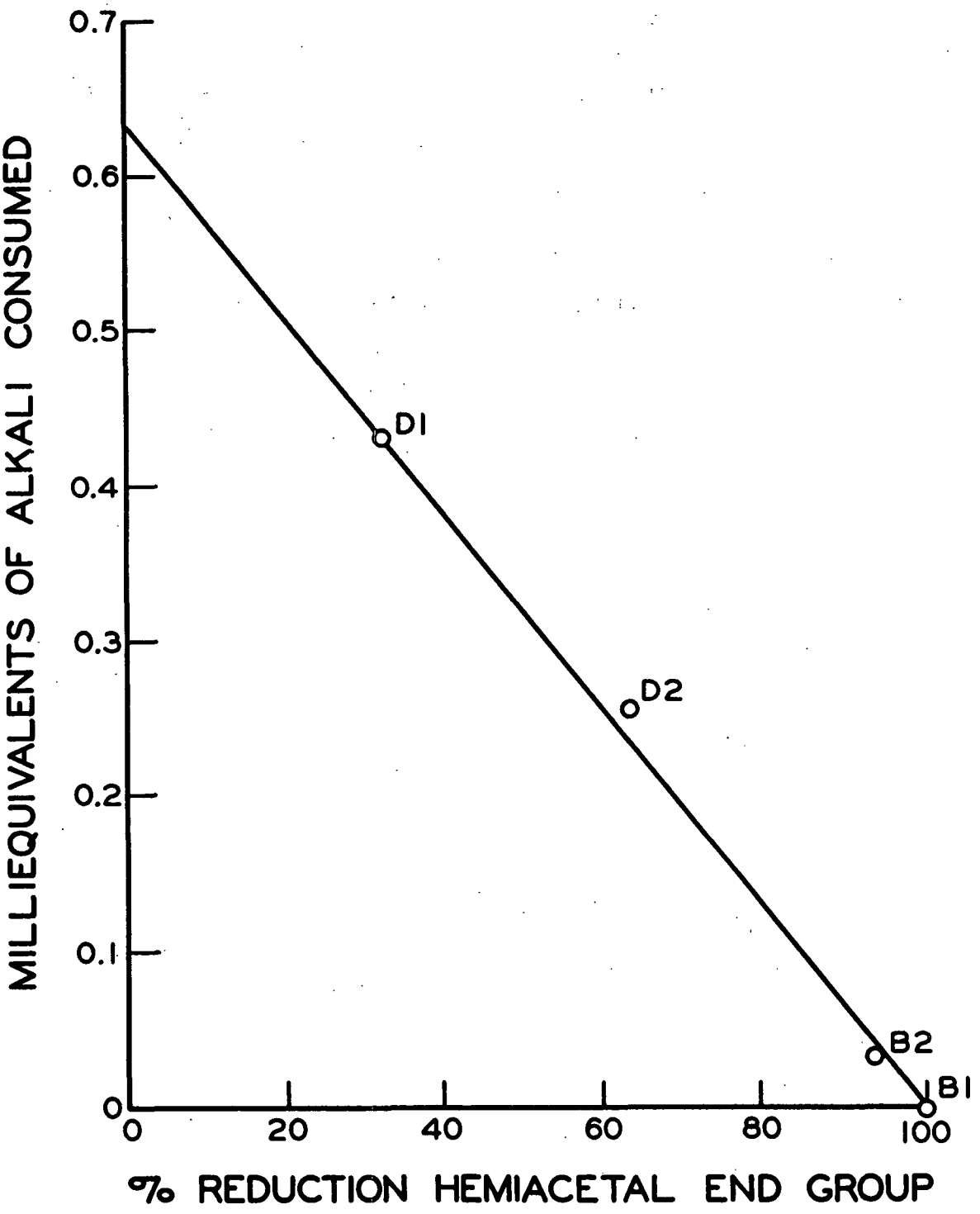


Figure 10. The Relationship Between Percent of Reduced Hemiacetal End Group and Alkali Consumption for the Reduced Polysaccharides

EXPERIMENTAL

PREPARATION OF ALGINIC ACID

ISOLATION OF ALGINIC ACID (31)

Alginic acid was isolated from the stipes of the brown algae, Laminaria hyperborea. The stipes were obtained from Girvan, Ayrshire, Scotland (Alginate Industries Ltd.) on January 20, 1965. The L. hyperborea was collected from the beaches and air dried for a period of several months. The stipes were oven dried to 90% solids and milled to pass an 8-mesh screen.

Three hundred grams of the stipes, screened to pass an 8-mesh screen and be retained on a 100-mesh screen, were immersed in 12 liters of 0.25% calcium hydroxide solution heated to 55-60°C. (The calcium alginate is insoluble.) After stirring for 30 minutes, the green-brown liquid was decanted and discarded. The stipes were washed five times with 10 liters of water.

Sulfuric acid (3.75 liters, 0.2N) was stirred with the stipes for 15 minutes and decanted. The same amount of sulfuric acid was again added to the stipes and the mixture was cooled to 4°C. The mixture was left stirring at this temperature for 12 hours. The acidic liquid was decanted and the residual stipes were again washed five times with 10 liters of water. The last wash was with deionized water.

The alginic acid was extracted by cooking the stipes at 40-60°C. for three hours with 7.5 liters of 3% sodium bicarbonate solution. The resulting brown jelly was diluted with 30 liters of deionized water and stirred for 12 hours at room temperature. The slurry was diluted further by adding another 20 liters of deionized water. The residual solids were allowed to settle for 2 hours and

the clarified liquor was decanted. Some of the residual solids were removed from the sediment by filtering through a 16-mesh screen. These solids were washed with 10 liters of water. The supernatant liquid and wash water were combined with the clarified liquor. The smaller residual solids were removed by centrifugation of the liquor at 15,000 g on the Beta-fuge* continuous flow head operated at 15°C.

The alginic acid was precipitated from the clear yellow solution by the addition of enough 1N HCl (approximately 4.5 liters) to bring the pH to 2.6. Haug and Larsen (16) have shown that this hydrochloric acid precipitation will separate alginic acid from polysaccharides containing glucose, xylose, and arabinose. The carbon dioxide evolved upon acidification caused the alginic acid precipitate to float to the top of the liquid. The clear lower liquid was siphoned off. The gelatinous alginic acid precipitate was removed by centrifugation with the Beta-fuge continuous head operated at 20,000 g. The precipitate was washed with 10 liters of 0.1N HCl and 10 liters of distilled water. After each wash, the alginic acid was recovered by centrifugation at 20,000 g. This procedure yielded 60.2 grams (ovendry content) of alginic acid.

BLEACHING OF ALGINIC ACID

The alginic acid precipitate was obtained as a light brown jelly which gave off the odor of decaying meat. To rid the alginic acid of this objectionable impurity, a mild room-temperature acid chlorite bleach was employed (32).

The entire alginic acid precipitate was dispersed in 4.5 liters of distilled water containing 6.0 grams of sodium chlorite and enough acetic acid to bring the pH to 3.8. After remaining at room temperature for 3 hours, the slurry was

*Beta-fuge, Model A, Lourdes Instrument Corp., Brooklyn, N. Y.

transferred to dialysis tubing. The dialysis tubes were placed in 30 liters of deionized water. The water was changed three times over a 20-hour period. The dialysis tubes and their contents were then placed in a moving air stream and about one third of the water was removed by pervaporation. The dialysis tubes and contents were frozen to -20°C. and the dialysis membrane was removed. The alginic acid was recovered as a white sponge by the freeze-exchange* process through two acetone washes and one petroleum ether (b.p. 30-60°C.) wash. The alginic acid sponge was air dried.

PREPARATION OF SODIUM ALGINATE

Since alginic acid is insoluble in water, it was found necessary to convert a part of the alginic acid to its water-soluble sodium salt.

A portion of the alginic acid (5.00 g. airdry) was placed in a 400-ml. beaker containing a magnetic stirring bar. The alginic acid was dispersed by stirring in 200 ml. of water. Enough 1N NaOH was added to bring the pH to 11. The clear, highly viscous solution was dialyzed for 68 hours to remove the excess salts. The sodium alginate was recovered as a white sponge by the freeze-exchange process through acetone and petroleum ether.

This sodium alginate was used for determination of optical rotation, intrinsic viscosity, and the phenol-sulfuric acid color reaction.

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*Freeze-exchange is used to designate a new recovery procedure. The frozen alginic acid slurry was dropped into 2 volumes of chilled (4°C.) acetone. The temperature of the ice-acetone mixture dropped below zero and was kept there for a period of four hours by insulating the containers. The alginic acid was recovered as a hydrogen-bonded sponge which could be easily handled, drained, washed with nonpolar solvents and air dried. A patent has been applied for this process.

FRACTIONATION OF SODIUM ALGINATE

Haug (18) has reported a fractionation procedure which allows separation of alginates with different uronic acid compositions. Molecules containing a large portion of guluronic acid were accumulated in the fractions which are soluble in potassium chloride and are precipitated by manganese ions, whereas molecules rich in mannuronic acid are accumulated in the opposite fraction.

In an attempt to further enrich the L-guluronic acid content of alginic acid, an exploratory potassium chloride precipitation procedure was carried out. However, there was no precipitate formed when the sodium alginate from L. hyperborea was used, even when an excess of potassium chloride was added.

ANALYSIS OF ALGINIC ACID POLYMERS

MOISTURE

The moisture was determined by Institute Method 3. This involves finding the weight loss upon heating at 105°C. for 16 hours.

ASH

Two (1,000 g. airdry basis) alginic acid samples were weighed into tared crucibles. The samples were ignited slowly and heated at 600°C. for 4 hours. The crucibles were cooled in a desiccator over P_2O_5 and weighed. In order to correct for carbonate, each of the ash samples was dissolved in 25.00 ml. of 0.100N HCl. The solutions were heated to boiling, cooled rapidly to 50°C., and titrated to the phenolphthalein end point. The basicity is due to loss of carbonate, so ash is expressed as ash minus carbonate.

ANHYDROURONIC ACID

The anhydrouronic acid was determined by analysis of the CO_2 evolved upon distilling the alginic acid polymer in 19% hydrochloric acid (72). Much more reproducible results are obtained using 19% HCl for a polymer having such a high uronic acid content. This test was performed by the Analytical Department of The Institute of Paper Chemistry.

In addition to analysis by CO_2 evolution, the anhydrouronic content was determined by titration (71). A sample of alginic acid (500 mg. ovendry basis) was weighed into a 250-ml. beaker. A gram of sodium chloride was added in order to sharpen the end point. One hundred milliliters of distilled water were added slowly to the mixture while stirring continuously on a magnetic stirrer. The alginic acid was titrated with standard alkali (0.100N NaOH) at a rate such that the pH, read with a glass electrode pH meter, never exceeded 8.5. The alkali was added until a pH of 7.5 persisted for 30 seconds. The equivalents of alkali consumed are equal to the equivalents of anhydrouronic acid carboxyl.

ESTERIFICATION

To the neutral solution obtained from anhydrouronic acid titration was added sufficient alkali to cause complete deesterification of the polymer (6.0 ml. of 1.00N NaOH for degree of substitution <0.5; 10.0 ml. of 1.00N NaOH for degree of substitution >0.5).

The equivalents of alkali consumption are equal to the equivalents of ester present (71).

Since titration for esters is not accurate at high degrees of esterification, the hydroxylamine-ferric perchlorate color test for esters was adopted (35). The

reagents for the analysis were prepared the same as described by McComb and McCready (35) except that the stock ferric perchlorate solution was prepared from 3.27 g. of $Fe(ClO_4)_3 \cdot 6H_2O$ obtained from G. Frederick Smith Chemical Co., Columbus, Ohio. These crystals were dissolved in approximately 50 ml. of water containing 2.0 ml. of 70% perchloric acid. The resulting clear solution was diluted to 100 ml. in a volumetric flask and stored in a refrigerator where it is stable for one month.

Crystalline β-D glucose pentaacetate was used as the standard exactly as described (35). The acetyl content was converted to an equivalent n-propionyl content by using the information found in Thompson's article (73). He found that at the same molar concentration (8 nm. x 10^{-4} M) acetohydroxamic acid – ferric perchlorate complex absorbed less at 520 nm. than n-propionohydroxamic acid – ferric perchlorate complex: 0.365 to 0.380, respectively. Using this correction and the correction for the difference in molecular weight between acetyl and n-propionyl, a standard curve for n-propionyl was obtained.

The method for analyzing the propionyl algin consists of weighing a sample of polymer estimated to contain 2-13 mg. of propionyl into a 100-ml. beaker. While stirring, 25.0 ml. of hydroxylamine solution were added. Into the dropping funnel was placed 25.0 ml. of sodium hydroxide solution. This was added dropwise with stirring over a 15-minute period. Stirring was continued until the polymer dissolved. Two milliliters of the solution were pipetted into a 25-ml. volumetric flask. To this was added 5 ml. of water and 5 ml. of acid – methanol, and this was mixed thoroughly. The solution was made to volume with the addition of the ferric perchlorate solution with continuous mixing. The solution was allowed to stand for five minutes. The solution was filtered through Whatman No. 42 paper into a 50-ml. filter flask in an ice bath. Filtration took place under 10 inches Hg vacuum. These conditions were used to minimize loss of methanol.

The filtrate was analyzed for absorbance at 520 nm. with a Beckman DU spectrophotometer using 1-cm. silica cells. The filter paper was observed for the existence of a red precipitate which would be indicative of algin hydroxamic acid formed from esterified uronic acid carboxyls.

SUGAR ANALYSIS

The composition of the alginic acid from L. hyperborea stipes was determined by paper chromatography of an acid-hydrolyzed alginic acid. The alginic acid was hydrolyzed exactly as recommended by Haug and Larsen (33). This involves hydrolysis in 80% sulfuric acid at 20°C. for 18 hours, followed by hydrolysis at 100°C. for 5 hours in 2N H_2SO_4. Neutralization was carried out by adding a slight excess of $CaCO_3$ to the hydrolyzate after cooling. The precipitate of $CaSO_4$ was removed by filtration.

To convert the lactone fraction to the sodium salt, half the hydrolyzate was titrated with 4N NaOH to a red color with phenolphthalein. After standing for one hour, the solution was neutralized with a drop of glacial acetic acid.

The paper chromatographic procedure of Fisher and Dörfel (13) was used to analyze the hydrolyzates. For the separation of the uronic acids, the mixed solvent (5:5:1:3v/v) pyridine – ethyl acetate – acetic acid – water was used as the mobile phase. The liquid used to condition the sheets was 500 ml. of the mixed solvent (11:40:6v/v) pyridine – ethyl acetate – water. The lactones were separated by using only the (11:40:6) solvent for both the mobile and conditioning phases. The mixed solvent (8:2:1v/v) ethyl acetate – pyridine – water was used to analyze for neutral sugars. The chromatograms were air dried and sprayed with p-anisidine hydrochloride (0.5% in n-butanol) for the detection of reducing sugars or hydroxylamine – ferric chloride for the detection of lactones or esters (74). The compounds in the hydrolyzate were identified by comparison of the

mobility to known sugars such as gulose, mannose, xylose, glucuronic acid, and glucuronic lactone.

Haug and Larsen (33), in using this hydrolysis procedure on solutions of known uronic acid composition, have shown that guluronic acid decomposes more readily than mannuronic acid. The ratio of mannuronic to guluronic acid was multiplied by 0.66 to correct for the different rates of acid decomposition (33).

NUMBER AVERAGE DEGREE OF POLYMERIZATION

The number average degree of polymerization ($\bar{DP}_n$) of the alginic acid polymers was determined from the intrinsic viscosity of a sodium alginate solution. Donnan and Rose (17) have shown that sodium alginate forms a stable solution in a 0.1N NaCl and acetate buffer at pH 5.50. In addition, they found that there is a linear relationship between intrinsic viscosity in this solvent and $\bar{DP}_n$. Hence, by determining the intrinsic viscosity of sodium alginate in the buffer, its $\bar{DP}_n$ may be calculated by the following equation:

$$\bar{DP}_n = 58\ [\eta].$$

In this equation, $[\eta]$ is the intrinsic viscosity expressed in dl./g.

The intrinsic viscosity was determined in an Ubbelohode No. 75 capillary dilution viscometer at 30.0°C.

PREPARATION OF DI-O-PROPIONYL ALGINIC ACID

A modification of the propionation procedure of Carson and Maclay (39) was employed in order to achieve complete propionation in a shorter time.

FIRST PROPIONATION

Formamide (175 ml.) and pyridine (225 ml.) were added to a one-liter resin kettle and heated to 40°C. While stirring, freeze-exchange alginic acid (15 g.

air dried) was added and the mixture was held at 40°C. while stirring for 90 minutes. After the slurry had cooled to 35°C., propionic anhydride (200 ml.) was added over a 4-hour period. The reaction was left stirring for 17 hours at room temperature.

The reaction was terminated by precipitation into N HCl (5 liters at 4°C.). The polysaccharide was recovered by centrifugation, washed with one liter of water, one liter of acetone, and one liter of petroleum ether (b.p. 30-60°C.). The product was air dried with forced air for one hour and put through a flour sifter to obtain a uniform particle size.

This procedure was repeated on two more batches of alginic acid, and all the products were combined.

SECOND PROPIONATION

Because of the low degree of substitution, a second propionation was conducted on the product from the first propionation. The only difference from the first procedure is the order of addition of the solvents. The partially propionated alginic acid (20 g. air dried) was added to formamide at 40°C. To the thick paste was added pyridine at 40°C. After stirring for 90 minutes, the solution had cooled to 35°C., and propionic anhydride was added over a 4-hour period. The recovery procedure was the same as in the first propionation.

The second propionation was conducted on another 20 g. of partially propionated alginic acid and the two batches were combined.

PREPARATION OF METHYL DI-O-PROPIONYL ALGINATE

A portion (6.00 g. air dried) of di-O-propionyl alginic acid was placed in a 400-ml. beaker and stirred to a thick paste with the addition of 150 ml. of

tetrahydrofuran. The diazomethane was prepared according to the procedure of De Boer and Backer (75). The diazomethane was in a solution of 160 ml. of diethyl ether and 35 ml. of tetrahydrofuran. To this cooled (-60°C.) diazomethane solution was added the di-O-propionyl alginic acid dissolved in tetrahydrofuran. The polymer formed a fine, glassy precipitate. The slurry was stirred for 90 minutes at -60°C. The algin product was recovered by precipitation into 500 ml. of petroleum ether (b.p. 30-60°C.), filtered, washed with 500 ml. of the petroleum ether, and air dried at 50% relative humidity for 19 hours.

REDUCTION OF ALGIN POLYMERS

WITH DIBORANE GENERATED *IN SITU*

Di-O-propionyl alginic acid was reduced with diborane generated *in situ* according to the general procedure of Smith and Stephen (3). The reduction was conducted in a dry nitrogen atmosphere using the apparatus described by McKee (76). Sodium borohydride (11.9 g.) was dissolved in 300 ml. of "diglyme" [bis (2-methoxyethyl) ether] by stirring overnight at room temperature. The solution was added to a 1-liter resin kettle in a constant-temperature bath at 26°C. The resin kettle was swept by a dry nitrogen flow of 160 cc./min. for 1 hour. Di-O-propionyl alginic acid (10 g. airdried basis) was added to the diglyme solution and stirred for 30 minutes. The nitrogen flow was slowed to 43 cc./min., and the boron trifluoride etherate (52 ml. in 40 ml. diglyme) was added dropwise over a 2-hour period. During the addition of the boron trifluoride, the polymer slurry was stirred vigorously. After all the boron trifluoride had been added, the nitrogen flow was slowed to 7.5 cc./min. and the kettle contents were left stirring for 20 hours at 26°C.

The reduced algin polymer was recovered by precipitation into two volumes of petroleum ether (b.p. 30-60°C.). The precipitate was recovered by filtration

and dried with a flow of dry nitrogen of 200 cc./min. for 1 hour. The white granular solid was added slowly to 200 ml. of water containing 35 ml. of saturated sodium bicarbonate solution at 4°C. After about 5 minutes, no more gases were being evolved, so the slurry was brought to pH 7.4 with the gradual addition of saturated sodium bicarbonate solution. The salts were removed from the polymer by dialysis for 68 hours.

The dialyzed polymer slurry was concentrated by pervaporation to one third of its original volume (200 ml.). The reduced polymer was recovered by freeze drying.

WITH DIBORANE GENERATED EXTERNALLY

Di-O-propionyl alginic acid (14 g. air dried) was reduced with diborane generated externally according to the procedure of Ross and Thompson (6). Diborane was generated in the external flask by the addition of boron trifluoride etherate (73 ml.) dropwise over a 4-hour period to a solution of sodium borohydride (16.8 g.) in 150-ml. bis (2-ethoxyethyl) ether. The diborane gas was carried by a flow of dry nitrogen, 7.5 cc./min., through a trap containing a plug of glass wool, to the di-O-propionyl alginic acid in 420 ml. diglyme at 26°C. Using these conditions, the concentration of diborane in the polymer solution was the same as for diborane generated in situ.

After reacting for 20 hours, the polymer from the reduction was recovered by the sodium bicarbonate recovery procedure.

WITH LITHIUM BOROHYDRIDE

Lithium borohydride was obtained as a white powder with a minimum purity of 96% from the Callery Chemical Company, Callery, Pennsylvania. Since anhydrous lithium borohydride reacts explosively upon exposure to moist air, its solution

was prepared in a dry nitrogren atmosphere. The dry nitrogen atmosphere was easily provided by placing solvent, solute, and a balance in a large polyethylene bag equipped with arm ports. The bag was evacuated and filled with purified dry nitrogen. The transferring and weighing took place in this atmosphere.

Methyl di-O-propionyl alginate (5.44 g. air dried) was reduced with lithium borohydride by the same procedure which Rees and Samuel (4) used for the reduction of methyl di-O-acetyl alginate. The alginate polymer was dissolved in 200 ml. of tetrahydrofuran by stirring for 2 hours at room temperature. The solution was transferred to a one-liter three-necked, round-bottom flask and placed in an oil bath at 80°C. under total reflux. Immediately, the lithium borohydride solution (approximately 8 g. in 200 ml. of tetrahydrofuran) was added dropwise over a 90-minute period. The slurry quickly formed a gel which was left overnight. After reacting for 18 hours, the gel was broken and a white gelatinous precipitate had formed.

The flask was cooled and water (200 ml.) was added slowly over a 3-hour period to react with excess lithium borohydride. Even though caution was used, some of the slurry bubbled over. The contents of the reaction flask were transferred to dialysis tubing and dialyzed for 48 hours. The polymer slurry was concentrated by pervaporation and the polymer recovered by freeze drying.

In an analogous manner, half of the polymer from the reduction with diborane externally generated was reduced with lithium borohydride. The concentration of the polymer and the lithium borohydride were kept the same as for the reduction of methyl di-O-propionyl alginate.

ANALYSIS OF REDUCED ALGIN POLYMERS

In some cases the method for the analysis of the reduced polymer was the same as for the analysis of the alginic acid polymers before reduction. Only

if the method of analysis was different for the reduced polymer will it be discussed in this section.

ANHYDROURONIC ACID

The anhydrouronic acid content of the reduced polysaccharides was obtained by titrating the CO_2 evolved from the polymer upon distillation from 12% HCl according to Institute Method 25. These analyses were performed by the Analytical Department of The Institute of Paper Chemistry.

TOTAL n-PROPOXYL

The total n-propoxyl content was determined by collecting the volatile alkyl iodides from the polymer boiling in hydroiodic acid.* The method is a modification of Institute Method 18 for methoxyl determination. The microburners were adjusted to reflux the reaction mixture vigorously enough so that the air condenser was hot to the touch within one inch of the first bend in the apparatus. In order to determine if carryover was complete, after one hour the bromine traps were removed and replaced with traps containing fresh bromine solution.

To check if the conversion of the n-propyl iodide and its subsequent distillation into bromine traps were quantitative, a sample of vanillin n-propyl ether was analyzed by the above procedure. A value of 31.79% volatile alkyl iodides, calculated as methoxyl, were obtained. A theoretical value of 31.95% would be expected. Thus, a yield of 99.5% was obtained.

In the case of the vanillin n-propyl ether as well as for all samples, the reaction and distillation were found to be complete at the end of one hour.

*Performed by the Analytical Department of The Institute of Paper Chemistry.

ALKALI-LABILE n-PROPOXYL

The polysaccharide (500 mg. ovendry content) was dispersed in 5.0 ml. of distilled water in a small (20-ml.) distillation flask. The flask was heated in an oil bath at 115°C. and 2.0 ml. of the neutral distillate were collected in approximately 30 minutes. To the flask was added 2.5 ml. of oxygen-free 4N NaOH. The flask remained in the oil bath until 2.0 ml. of the distillate from the alkaline solution had been collected.

The neutral and alkaline distillates were analyzed for organic compounds by the Hy-Fi gas chromatograph*. The columns were 5 ft. by 1/8 inch, either 20% Carbowax 2000 with terephthalic acid 60/80 mesh or a 20% Carbowax 20m column containing diethylene glycol succinate 60/80 mesh. The following conditions were used at all times: nitrogen 34.8 ml./min. (10 lb. pressure), injector set at 40 (128°C.), oven set at 100 (90°C.), and hydrogen flow of 30 ml./min.

Typical retention times are shown in Table V.

The unknown compounds in the distillate were identified by their relative retention times and by mixture with known solutions. The amount of n-propanol in the distillates was determined by the area under the peak on the gas chromatogram.

FORMATION OF n-PROPYL ACETATE

Ten milliliters of the aqueous alcohol solution were transferred to a 50-ml. separatory funnel and saturated with potassium carbonate. The solution was extracted with 5 ml. of diethyl ether, and the aqueous layer was discarded. To the ether extract was added 1 ml. of acetyl chloride and one drop of 70% perchloric acid. The reaction took place at room temperature for 15 minutes.

*Aerograph Hy-Fi Model 600, Wilkens Instrument and Research, Inc., Walnut Creek, California.

TABLE V

GAS CHROMATOGRAPHIC RETENTION TIMES[a]

Retention Time, min.	Compound
0.8 and 1.0	petroleum ether (b.p. 30-60°C.)
1.1	acetone
1.4	tetrahydrofuran
1.8	isopropanol
2.0	ethanol
3.0	n-propanol
5.8	n-butanol
6.1 to 9	n-propionic acid
not removed in one hour	amylamine
not removed in one hour	pyridine

[a]Carbowax 2000 column was used.

The reaction was terminated by adding 10 ml. of saturated aqueous potassium carbonate slowly to the reaction, and the two immiscible liquids were shaken for 2 minutes. The excess acids liberated carbon dioxide, and some ether evaporated, so an additional 2 ml. of ether were added. The ether-saturated K_2CO_3 mixture was shaken and the ether solution was recovered.

The ether solution was analyzed by the Hy-Fi gas chromatograph under the conventional conditions and showed only n-propyl acetate to be present. The ether extract gave a positive test for esters by the hydroxylamine – ferric chloride test (79).

ALKALI CONSUMPTION

A portion of each of the polysaccharides (93 mg. ovendry content) was placed in a 10-ml. volumetric flask and flushed with a flow of dry nitrogen (8 liters/hour) for five minutes. To the polysaccharide was added nitrogen-purged sodium hydroxide (5.0 ml., 0.50N). The polysaccharide was dispersed by shaking for 15 minutes at room temperature (25°C.) in the stoppered flask. The stoppered flask was placed in an oil bath at 90°C. for thirty minutes. The reaction was terminated by placing the flask in cold (8°C.) water, and the solution was neutralized with sulfuric acid (5.0 ml., 0.500N). The flasks were stoppered overnight in a refrigerator at 6°C. The following morning, the contents of each of the flasks were transferred to a 100-ml. beaker. The slurries were stirred and titrated with 0.050N NaOH to a pH of 8.00. Each value of the alkali consumption was corrected for a blank and for n-propionyl ester content.

NUMBER AVERAGE DEGREE OF POLYMERIZATION

The number average degree of polymerization ($\bar{DP}_n$) was determined on the polysaccharides by the osmotic pressure of their triacetate derivative. The acetate derivative was chosen because of the extensive work of Linnell (77), who optimized the acetylation conditions and explored solvents for osmometry of black spruce glucomannan triacetate.

Linnell's acetylation procedure (77) was performed on 250 mg. (ovendry basis) of each of the reduced polysaccharides. The polymer was dispersed in formamide (4.4 ml.) by stirring over a 3-hour period. Pyridine (8.1 ml.) was added while stirring during a 2-hour period. Acetic anhydride (6.25 ml.) was added dropwise to the polymer in the pyridine – formamide solvent over a 3-hour period. The reaction was stirred continuously for two days and allowed to sit for two more days at room temperature (25°C.). The acetylated polysaccharide

was precipitated into 5 volumes of cold (5°C.) N HCl and methanol mixture (10% methanol by volume). The precipitate was recovered by centrifugation in the Beta-fuge operated at 8°C. and a field of 15,000 g. The precipitate was washed with cold 0.1N HCl and distilled water and freeze dried. Analysis of the acetylated polysaccharides by the hydroxylamine – ferric perchlorate method of McComb and McCready (35) showed all the polysaccharides to be completely acetylated.

The $\bar{DP}_n$ was determined with a Mechrolab High Speed Membrane Osmometer, Model 501, operated at 25°C. on the acetylated polysaccharide in 1,1,2-trichloroethane. Schleicher and Schuell number 07 membranes (Keene, New Hampshire) with an average pore diameter of 5 to 10 nm. was employed. The membranes were conditioned by solvent exchange.

The number average degree of polymerization was calculated according to the procedure described in the operation manual for the osmometer (78). The $\bar{DP}_n$ was obtained by dividing the molecular weight by 288, the weight of an anhydrotriacetate hexoside.

QUALITATIVE SUGAR ANALYSIS

Each of the reduced polysaccharides (50 mg. air dried) was hydrolyzed for 5 hours in N H_2SO_4 (3.3 ml.) at 95°C. The hydrolyzate was neutralized with $CaCO_3$, and the $CaSO_4$ was removed by filtration. The precipitate was washed with 4 ml. of distilled water. Half of the hydrolyzate was neutralized with N NaOH to a phenolphthalein end point for one hour to open any lactones. The neutralized and acidic fractions were analyzed for uronic acid and lactones exactly as described on p. 55 for the analysis of alginic acid. These neutral and acidic fractions were also run in (9:1:1v/v) saturated with boric acid, methyl ethyl ketone – acetic acid – water for the separation of mannose and

gulose (5). The chromatograms were developed by spraying with p-anisidine HCl (5% in n-butanol).

For comparison, authentic samples of mannose, gulose, glucose, glucuronic acid, and glucuronic lactone were developed on the chromatogram. The compounds in the hydrolyzate were identified by their R_G, by reaction with sprays, and by comparison to known compounds.

DEESTERIFICATION WITH HYDROXYLAMINE

In order to remove esters from the algin polysaccharides without causing alkaline degradation to the polymer, the very mild hydroxylamine deesterification procedure was developed.

The algin polymer (2.50 g. airdry basis) was dispersed in 100 ml. of water at 20°C. by stirring. Hydroxylamine hydrochloride (28 ml., 2M) was added to the slurry and the pH was brought to 8.0 with the slow addition of sodium carbonate (approximately 70 ml., 1N). After stirring for two hours at room temperature, the slurry was dialyzed for 68 hours. The water outside the dialysis tubing showed a loss of less than 1% carbohydrates by the phenol – sulfuric acid color reaction (33).

The dialyzed algin polymer was concentrated by pervaporization to approximately 75 ml. water. The remaining water was removed by either freeze drying or freeze-exchange through acetone and petroleum ether (b.p. 30-60°C.).

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